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Experimental Cell Research, **6**, *255–262* (*1954*)

MICROSCOPIC AND SUBMICROSCOPIC STRUCTURE OF THE SYNAPSIS IN THE VENTRAL GANGLION OF THE ACOUSTIC NERVE[1]

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Received June 9, 1953

A study of the synapsis by means of the electron microscope is of great interest to elucidate a series of controversial points referring to its structure, as revealed by optical methods. This study also attempts to obtain information regarding the submicroscopic organization of the site where the synaptic transmission takes place.

One of these controversial problems refers to the existence of a structural material interposed between the two membranes of the contacting neurons. Recently in a general report on "The nature and types of synapsis" one of us (9) postulated, on the basis of microscopical and experimental observations, that there is no material interposed at the level of the synaptic junction.

Another of the controversial points debated for a long time refers to the continuity of the neurofibrillar material at the synapsis. However, the results of the experimental degeneration of the presynaptic fiber accompanied by hypertrophy, hyperargentophilia and detachment of the neuropodia and other nervous endings, demonstrate that at the level of the synapsis two independent neurofibrillar and neuroplasmic systems exist (7, 8).

The submicroscopic study of the synapsis can be carried out by means of ultrathin sections examined with the electron microscope. However, the interpretation of the electron micrographs of nerve cells and endings is hindered by the extreme thinness of the section in relation to the volume of the cells. This difficulty is still increased by the complex spatial relations existing between the nerve endings and the surface of the cells and dendrites.

In this work we have to some extent tried to obviate these difficulties and to establish, as far as possible, a correlation between the optical and the electron microscope images of the synapsis. With this purpose we have compared the results obtained in ultrathin sections (0.1 to 0.2 μ) examined under the electron microscope with the observation of silver-stained thin sections (0.5 to 1 μ) of the same tissue under the optical microscope.

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[1] Work supported by a grant of the Rockefeller Foundation.

For the comparative study of the microscopic and submicroscopic structure of the interneuronal synapsis we have selected the ventral ganglion of the acoustic nerve. This selection was based on the following facts: 1) it is a highly differentiated primary nervous center containing a wide variety of specific synapses in addition to more primitive synaptic elements such as the end-feet found in the spinal cord—neuropodia, "Endfüße of Held and Auerbach"; 2) good silver-stained, *in toto*, preparations of this material can be obtained regularly; 3) the tissue fragments fixed and embedded to be examined with the electron microscope are comparable with similar fragments fixed and stained with silver; 4) the terminal fibers coming from the ganglion of Corti and contacting each cell are thick and only three or four in number; 5) this material is excellent for showing experimental changes in the synapsis.

TECHNIQUES

Fragments of the ventral acoustic ganglion from young dogs and cats were fixed in a solution of 2 per cent osmic acid in acetate veronal buffer having a pH of 7.4 according to the procedure described by Palade (13). In some instances this fixation was followed by a treatment with formaldehyde at pH 7.4. This material was embedded in a mixture of methyl and butyl methacrylate as described by Newman, Borysko and Swerdlow (12) with slight modifications. Both the thin and ultrathin sections were made in a special thermal expansion microtome designed at the Rockefeller Institute in New York. The sections were observed without removal of the embedding. Electron micrographs were taken at magnifications ranging from 1700 to 3200 × with an R.C.A. EMU 2 C electron microscope provided with a "wide field" objective pole piece as described by Hillier (10). The contralateral ganglion was simultaneously fixed in formalin and stained with silver according to Cajal's technique as modified by Estable (unpublished). This material was also embedded in methacrylate and sections of it from 1 to 0.5 μ were examined under the optical microscope.

RESULTS

Some of the wide variety of synaptic types present in the ventral acoustic ganglion are represented in Figs. 1, 2 and 3. These are thick silver-stained sections (30 μ) where the end-feet, club-endings and the more complex bulbs of Held from the nerve terminals are in intimate contact with the cell surface. Fig. 4 shows a thin silver-stained section of about 1 μ. Typical club-endings and end-feet are seen surrounding the cell and the dendrites. The differential impregnation of the nerve endings shows that these cells are almost completely surrounded by end-feet and club-endings that constitute the axonic component of the axo-somatic cochlear synapsis. The space separating the outer limit of the cell and some of the nerve endings can be explained by retraction phenomena caused by the fixatives and by the embedding procedure. A reticular appearance is frequently observed within the expanded portion of the nerve terminal. The observation of silver-impregnated thin sections of this material enables us to demonstrate already with the optical microscope that this appearance results from the precipitation and conglomeration of large silver granules. Nevertheless, in less heavily impregnated sections the neurofibrillar material does not show this granular aspect under the optical microscope.

The electron micrograph of Fig. 5 shows an ultrathin section of a nerve cell surrounded by nerve endings. In spite of the artificial fissure shown at the left portion of the cell cytoplasm, it can be observed that all the nerve terminals are in close relationship with the cell surface. Two thick endings are seen at the left, one of them having the typical club-like appearance with a width of 3 to 4 μ at the end. Most of the others appear to belong to the smaller end-foot type whose width ranges between 0.5 to 2 μ. The narrower zone of the end-foot which follows the preterminal portion of the nerve endings can only be seen in a few cases. Most of the end-feet appear in the sections as circles or ovoids surrounded by a membrane having a high electron density. This membrane, which also surrounds the preterminal portion of the nerve ending, is the only element separating the cytoplasm of the nerve cell and the presynaptic axoplasm. The thickness of this membrane as measured on this electron micrograph ranges between 2000 Å for the thick club-shaped endings to 330 Å for the smaller buttons.

The submicroscopic characteristics of the synaptic membrane and the adjoining pre- and postsynaptic cytoplasm can be better observed in Fig. 6. This is an electron micrograph of a subtangential section of the edge of a large nerve cell. This cell is seen to come into contact with three thick club-shaped endings and several smaller ones which, probably on account of the slant of the section, appear as embedded and surrounded by the cytoplasm. The synaptic membrane stands out clearly due to its high electron density, and its thickness is seen to vary in the different terminals. It ranges from 1600 to 2000 Å in the thick club-endings to 320 and 800 Å in the smaller end-feet. It is interesting to point out the fact that the thinner membranes show a double-edged appearance probably corresponding to the two membranes coming into contact. The presynaptic protoplasm has a low electron density and it appears to contain only submicroscopic protofibrils similar to the fibrillar substructures of Richards, Steinbach and Anderson (15), filaments of Schmitt (17) and the ones described by Fernández-Morán (11) and afterwards by De Robertis and Franchi (6). These protofibrils are few in number and large clear spaces are seen between them. They run more or less parallel to the fiber axis and are in many instances seen to end exactly at the level of the synaptic membrane. In the presynaptic zone of some terminals ovoidal granules with a mitochondrial appearance are also seen. In some zones the presynaptic protoplasm appears to be detached from the synaptic membrane and a line about 100 Å thick can be observed. This line probably represents the axon membrane (6).

The appearance of the nerve cell cytoplasm close to the synaptic junction is quite different from that of the nerve terminal. It has a more compact structure and its electron density is much higher. Our observations of this structure are not numerous and shall not be described here. We shall only point out the fact that a number of mitochondria and other smaller granules resembling them are seen in the neighborhood of the synapsis. All of these bodies appear ovoidal in the sections. They show a clear external membrane and a lamellar internal structure similar to the one described in the mitochondria of other cell types.

DISCUSSION

According to our information only preliminary observations have been published up to date on the synapsis as seen under the electron microscope. These studies refer to invertebrate synapses (16) and to synapses found in the brain stem of vertebrates (14). The work of Robertson (16) corresponds to a very special kind of axo-axonic synapsis in the crayfish and in the squid which is not the typical type of synapsis neither in the invertebrates nor in the vertebrates. He states that the minimal distance existing between the pre- and postsynaptic axoplasm is of 600 Å. Pease (14) points out the high water content of the axoplasm at the level of the terminal enlargement and the fact that the surface of the end-feet comes in direct contact with the surface of the nerve cells.

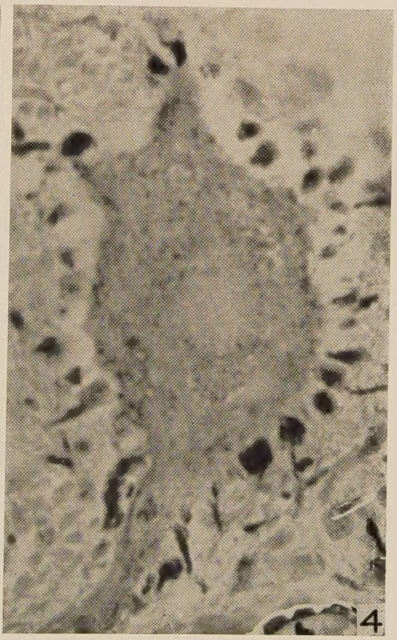
Figs. 1 to 4 are photomicrographs of silver-stained sections of the ventral acoustic ganglion of young dogs. Figs. 1–3 are thick sections of about 30 μ thick. Fig. 4 is a thin section of about 1 μ thick.

Fig. 1. Surface view of the pericarion of a ganglionic cell showing the numerous nerve terminals of various types (buttons, clubs, and rings) which end at the surface of the cell. 1,000 ×.
Fig. 2. Equatorial view of the same cell as in Fig. 1. The edge of the cell is completely surrounded by nerve endings intimately bound to the cell surface. 1,000 ×.
Fig. 3. Four ganglionic cells showing the complex nerve endings known as Held bulbs, in intimate contact with the cell body. 7,175 ×.
Fig. 4. Thin section of a ganglionic cell (see description in text). 1,300 ×.

1

2

3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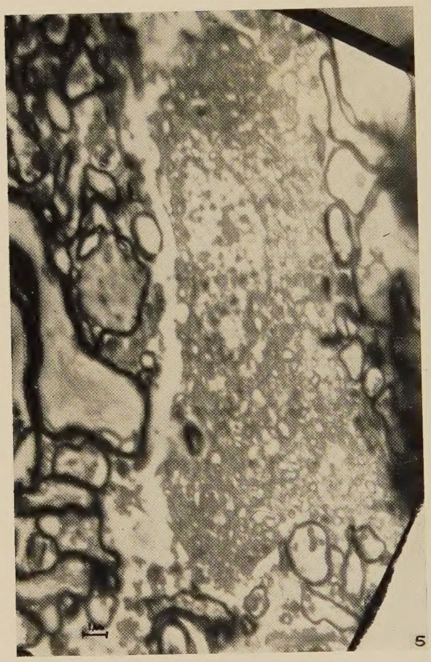
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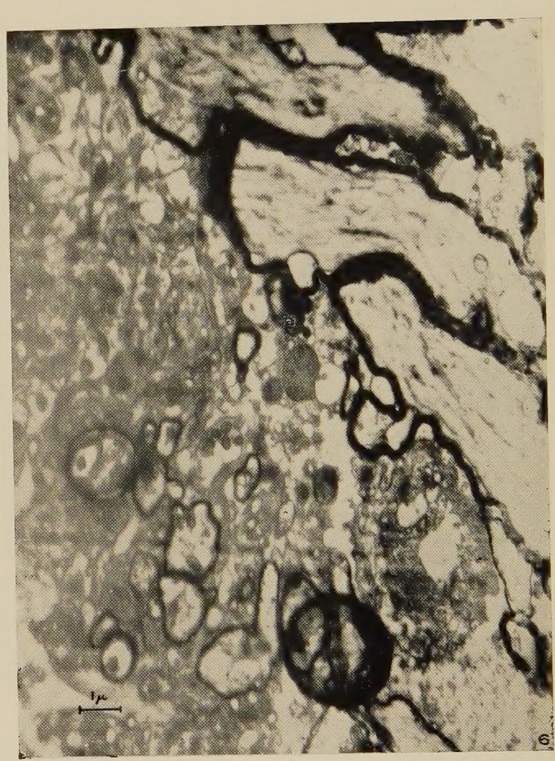
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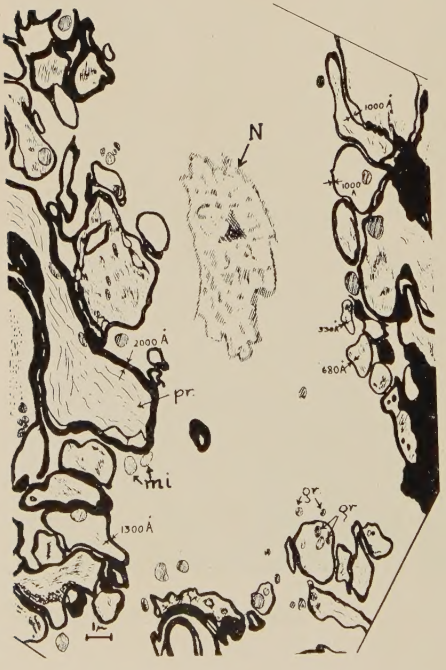
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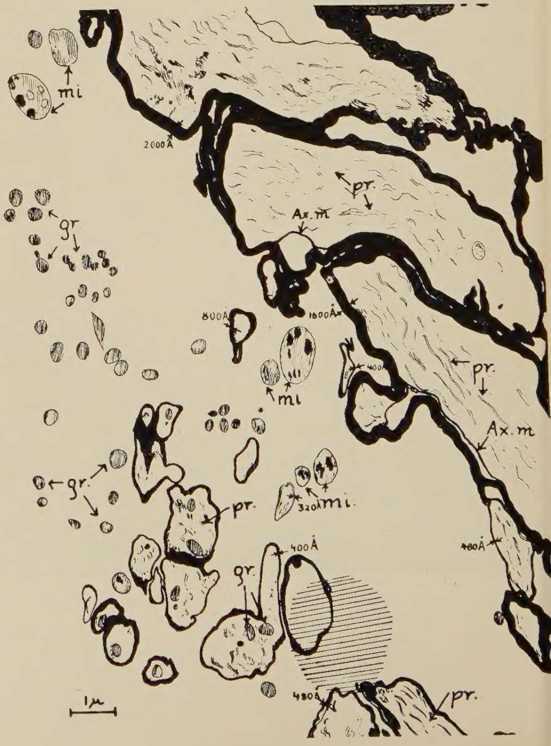
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gr.
480 Å
480 Å
pr.
1μ

According to our findings it can be shown that at the level of the synapsis the nerve terminals come into direct contact with the cell surface. The membrane interposed between the pre- and postsynaptic protoplasm has a high electron density and it is a continuation of the sheath of the nerve terminal. The electron density of this membrane is similar to that of the myelin sheath surrounding the nerve fibers although it is much thinner. It might be assumed, although nothing proves it that this membrane is continued as a submicroscopic structure in the nerve terminals and that it surrounds the synaptic end-feet and club-endings. Nevertheless, its thickness decreases gradually and very probably it tends to disappear at the smaller terminals, where the membrane separating the pre- and postsynaptic protoplasm has a double-edged appearance and a total thickness of only 330 Å. It seems very likely that at this level the two sheets represent the axon membrane and the plasma membrane of the cell coming into contact without the presence of any interposed material between them.

Another interesting fact arising from our observations is that at least in the synapsis of the acoustic ganglion between the nerve cell and the presynaptic terminal there is no interposed material alien to these two components. We are referring specially to the glial sheath postulated by some authors (3, 4). No doubt, the resolving power of the electron microscope would make it possible to visualize any interposed glial sheath, even if it were of submicroscopic dimensions.

The observation of a constant and neat separation between the pre- and postsynaptic protoplasm at the interneuronal synapsis confirms and extends to a submicroscopic level the neurone doctrine of Cajal (1, 2).

The existence of synapses where the separation between the pre- and postsynaptic protoplasm varies between such wide limits (330 to 200 Å) leads us to think that probably the different junctions may have different properties regarding the passage of the nerve impulse and the transfer of substances originating or playing a role in the process of synaptic transmission.

Fig. 5. Electron micrograph of an ultrathin section of a ganglionic cell of the ventral acoustic ganglion from a young dog fixed in 2 per cent osmic acid in veronal-acetate buffer at a pH of 7.4 for two hours and then in 10 per cent formaldehyde in the same buffer at pH 7.4 for 5 hours. Butylmethymetacrylate embedding (see description in the text). In the accompanying diagram the following details are indicated: *N* nucleus; *mi* mitochondria; *gr* granules of mitochondrial aspect; *pr* protofibrils; → ← thickness of the membrane at the synaptic junction. Magnification 3,300 ×.

Fig. 6. The same as Fig. 5 (see description in text). In the adjoining diagram the following details are indicated; *Ax.m* axon membrane; *mi* mitochondria; *gr* granules of mitochondrial aspect; *pr* protofibrils; → ← thickness of the membrane at the synaptic junction. Magnification 5,500 ×

SUMMARY

The ventral ganglion of the acoustic nerve was selected for a microscopic and submicroscopic study of the interneuronal synapsis.

In this work the electron microscope observations made on ultrathin sections of osmic acid fixed material were compared with silver-stained thick (30 μ) and thin sections (0.5 to 1 μ) of the same material. The microscopic observations show a wide variety of synaptic terminals including end-feet, club-endings and Held's bulbs surrounding almost completely the body of the cell and dendrites.

The electron microscope observation shows typical club-like endings and end-feet in intimate contact with the cell surface. At the level of the synaptic junction the pre- and postsynaptic protoplasm are separated by a membrane of high electron density which is a continuation of the sheath which surrounds the terminal. This sheath has the same appearance and electron density as the myelin sheath of the nerve fibers. At the synaptic junction the membrane varies in thickness between 2000 and 320 Å. Some of the thinner membranes have a double lamellar appearance.

The presynaptic protoplasm has a very low electron density and shows a few protofibrils and ovoidal granules of mitochondrial aspect. In some cases there are indications of an axon membrane about 100 Å thick.

The postsynaptic protoplasm is more compact and of higher electron density. Mitochondria and smaller granules are observed in the neighborhood of the synapsis.

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Experimental Cell Research, **6**, *263–270* (*1954*)

REPEAT DOSE X-IRRADIATION ON SINGLE-TYPE CELLS[1]

TIME INTERVAL OF IRRADIATION EFFECT

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Received June 10, 1953

This study is concerned with single-type cells, prespermatogonia and spermatogonia and the period of time for a) depopulation of spermatogonia, b) initiation of restoration by prespermatogonia, and c) recovery or return to normal frequency, following an acute dose or fractionated dose of x-irradiation.

In the preliminary studies, an acute dose of 138r was given to a partially shielded animal. This dose resulted in complete depopulation of spermatogonia in a period of 3–4 days. From this observation, our attention is centered on those single-type cells, the prespermatogonium, for observing the time that elapses before restoration of spermatogonia is cytologically apparent. This elapse of time may be termed the period of x-irradiation effect on prespermatogonia. The interval of time necessary for the restoration of spermatogonia to a normal incidence in the tubules, as derived from prespermatogonia, has been determined. This period may be considered as the restoration period.

When the testes are depopulated of spermatogonia, as a result of an acute dose of 1440r, the new generation of spermatogonia develops from prespermatogonia (4). In reference to depopulation of spermatogonia, it has been demonstrated that a fractionated dose of x-irradiation (two doses of 294r given four days apart) is equal in effect to a much higher acute dose (1440r) (5). It is not known whether the effectiveness of the two doses is related to the time interval selected.

In this work, the period of the irradiation effect and the time required for a restoration of spermatogonia derived from prespermatogonia is also determined after an acute dose of 300r and after repeat doses of 138r given at selected intervals.

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[1] This work was supported by Grant C-1478 from the United States Public Health Service and by the Department of Public Health, Pondville Hospital, Walpole, Mass.

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[3] Medical Nuclear Physicist, Cancer Research Institute, N. E. Deaconess Hospital, Boston, Massachusetts.

METHOD AND MATERIAL

For this investigation, a total of 212 young adult 20–25 gms., pure line C57 black mice from the Pondville Hospital Colony were used. The animals were restricted in celluloid boxes while being irradiated. Partial shielding was accomplished by placing sheet lead over the boxes leaving exposed in the radiation field only the general location of the testes. Radiation factors were: 100 KvP, 15 Ma, 2.6 mm Al Hvl, 77 r/min measured in air, at TSD of 32 cm. Two investigations have been carried out. The dose or doses given are as follows: for the first group, an acute dose of 138r or 300r; the second group, fractionated doses of 138r were given at one, two or eight day intervals. For each of the investigations, 4 animals were killed at the days post-radiation intervals as indicated in Table I. Both testes of each animal were fixed in Zenker's solution, microscopic slides prepared and stained with Harris' hematoxylin and eosin.

Numerals tabulated in Table I represent the incidence of appearance of prespermatogonia and spermatogonia. The method used for determining this incidence is described in Table I.

RESULTS AND DISCUSSION

The per cent of tubules showing spermatogonia or prespermatogonia at intervals following x-irradiation is shown in Table I.

The time period required for a) depopulation of spermatogonia, b) increasing incidence of appearance of prespermatogonia and c) the return to normal frequency of appearance of spermatogonia is shown in Table II. This data is in agreement with the words of Heller (2) on the radiosensitivity of spermatogonia. This depopulation of a single-type cell, the spermatogonium, in a period of 3–4 days after an acute dose of 138r places the spermatogonia in the category of one of the most sensitive mammalian cells. It would be of great interest to compare the rate of disappearance of lymphocytes with spermatogonia as these cells are in the same category of radiosensitive cells.

The first recognized evidence of restoration of germinal epithelium is the increase in incidence of prespermatogonia. For example, Table I shows that from the fourth to the fifth day after an acute dose of 138r, the prespermatogonia show an increase in incidence of 10 to 31. The radiosensitivity of the spermatogonia of the testis in contrast to the radioresistance of the more primitive prespermatogonia or basement epithelium was noted by Barrow and Tullis (1). These authors also recognized prespermatogonia as the source cells for restoration or repopulation.

A list of the literature on the effect of x-irradiation on the male generative system has been given by Heller (2).

TABLE I

Outcome of X-irradiation on spermatogonia in mouse.

Days post-radiation	0	1	2	3	4	5	6	7	8	9	10	12	14	15	16	20	21	28	35
Single Dose – 300 r																			
Per cent Tubules Showing Gonia	47	6	0	0		0		0		0	0								
Per cent Tubules Showing Prespermatogonia	0	7	12	12				18		24	45								
Single Dose – 138 r																			
Per cent Tubules Showing Gonia	47	18	12	9	0	0	0		0		0								
Per cent Tubules Showing Prespermatogonia	0	0	0	13	10	31	40		52		46								
Fractionated Doses, 138 r each, Total 276 r. Time between Doses, in Days, as follows:																			
1.																			
Per cent Tubules Showing Gonia	18	2	0	0	0		0		0		0			0			0		
Per cent Tubules Showing Prespermatogonia	0	0	4	7	10		10		19		22			38			52		
2.																			
Per cent Tubules Showing Gonia	12		0		0	0	0		0		0	0	0		0	0			
Per cent Tubules Showing Prespermatogonia	0		4		6	4	6		19		19	14	34		50	42			
4.																			
Per cent Tubules Showing Gonia	0	0	0	0	0		0		0										
Per cent Tubules Showing Prespermatogonia	10	12	4	4	9		15		17				36		38				
8.																			
Per cent Tubules Showing Gonia	0		0	0	0			0				0							
Per cent Tubules Showing Prespermatogonia	52		12	14	10			21				42							

Radiation Factors: Dose in Roentgens measured in air, 100 KvP, 15 Ma, 2.6 mm Al Hvl, 77 r/min. at TSD of 32 cm.

The procedure for determining the per cent of tubules showing spermatogonia and prespermatogonia was as follows: slides representative of the whole of both testes were studied at a magnification of ×430 and from these slides 100 fields were studied; each field is not a microscopic field but a carefully selected median cross section of a tubule. The incidence of the germinal cells in these median cross sections at various periods post-radiation is the basis of this method of sampling.

The conclusions drawn from our data was that spermatogonia were the first to be affected. This is now demonstrated to be true for spermatogonia but not for prespermatogonia.

Another general observation (2, 9, 6), is the regeneration of spermatogonia in x-irradiated testes before depopulation of all the germinal elements is reached, but it was not certain what cells were responsible (7). Regeneration or restoration of the seminiferous epithelium is indicated in our work to be from prespermatogonia (capsular cells, stem cells).

The term "prespermatogonia" is used by the authors in the sense of the stem cell from which new spermatogonia are derived when spermatogonial mitotic activity is not adequate to maintain a normal population of the testes, or when depopulation has been effected by other circumstances such as x-irradiation.

Our observations indicated that in the capsule there exists an undifferentiated stem cell as described by Heller (2), Barrow and Tullis (1), Spargo (10), Eschenbrenner (3), *et al.* This cell can transform into a spermatogonium but in the process may appear as a cell described as prespermatogonia, dust cells, capsular cells, stem cells or basement membrane cell. In this non active stage, these prespermatogonia, located within the capsule, are elongate cells, similar in appearance to connective tissue cells. Normally these cells are not clearly identifiable when observed at 450 × under the microscope, and because of this, the incidence of their appearance at this stage is listed as 0.[1] After depopulation of spermatogonia by x-irradiation the prespermatogonial cells become active and the time when the incidence of appearance increases can be determined. These prespermatogonia differentiate into spermatogonia. Therefore, that time interval between the beginning of restoration and the return of a normal frequency of spermatogonia represents a cell transition period. This period is also associated with a change from radioresistance to the characteristic radio-sensitivity of spermatogonia not correlated with mitotic activity (4).

The period of the effect of x-irradiation after an acute dose of 300r under the conditions of the experiments is 5–8 days (Table II). One can now determine the period of effect after two fractionated doses of 138r each totalling 276r.

It was not known what effect a second dose of 138r, would have on the time period if given at any period up to 4 days. Therefore, in three series of investigations, animals were given a second dose of 138r (totalling 276r) at 1, 2, or 4 days after the first dose. In every case it was found that the interval lasted for 8 days after the last dose (See Table II). This 8-day interval after the second dose is the same period of time as after an acute dose of 300r.

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[1] Zero is a relative term simply indicating that in 100 fields these prespermatogonia were not identified.

TABLE II

Outcome of X-irradiation on single-type cells in mice.

Dose	Depopulation of Spermatogonia	First Appearance of Restoration by Pre-Spermatogonia	Return to Normal Frequency of Appearance[1] of Spermatogonia
		Acute Doses	
138 r	3–4 Days	4 Days	7–8 Days
300 r	2 ,,	5–8 ,,	10 ,,
		Fractionated Doses, 138 r each, total 276 r	
		Time After Last Dose	
Time Interval Between Doses (In Days)			
1	2 Days	7–8 Days	18 Days
2	1 Day	8 ,,	16 ,,
4	0 ,,	6–8 ,,	16 ,,
8	2–3 Days	6 ,,	12 ,,

A testis may recover to a normal incidence of spermatogonia in the tubules 8 days after an acute dose of 138r. This is not complete recovery. If on the 8th day, a repeat dose of 138r is given, it might be expected that the 2nd dose would give a result that is a repetition of the first. However, by our method of tabulating results, it shows that instead of restoration starting at 4 days, it does not begin until the 6th day. No gross or microscopic morphologic changes were observed to explain this delay in restoration. These findings on a single-type cell recalls the clinical observations of Quimby and MacComb (8), in that skin recovers from the first irradiation much more rapidly than from subsequent doses. Our work suggests that the initiation of recovery was delayed which may contribute to those findings of Quimby and MacComb (8).

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[1] The procedure for determining the incidence of appearance of germinal cells was as follows: Slides representative of the whole of both testes were studied at a magnification of × 430 and from these slides 100 fields were studied; each field is not a microscopic field but a carefully selected median cross section of a tubule. The incidence of the spermatogonia in these median cross sections at various periods post-radiation is the basis of this method of sampling.

Factors of Irradiation: Dose in Roentgens measured in air, 100 KvP, 15 Ma, 2.6 mm Al Hvl, 77 r/min. at TSD of 32 cm.

The interval required for a return to normal frequency of spermatogonia is listed in Table II. This could be listed from the time of the last dose, from the time recovery starts or from the time of the first dose.

In Table II return to normal frequency is calculated from the time after the last dose. It can be seen that two doses of 138r each (totalling 276r) delays recovery from 18 to 16 to 12 days if given 1, 2, 4, or 8 days apart respectively. Only 10 days is required for recovery after an acute dose of 300r. From these data, two developments are revealed. The repeat dose of 138r (totalling 276r) has a prolonging effect on recovery time as contrasted with the acute dose of 300r. Also, the rate of recovery is different after each of the intervals used. For example, if a second dose of 138r is given one day after the first, it takes 18 days for recovery. If the second dose is given two or four days after the first, recovery takes place in 16 days. It only takes 12 days for recovery if the second dose is given 8 days after the first. It can also be seen that the rate of recovery is different after each dose if considered from the time recovery starts (Table I).

It would appear that a second dose given at an interval of one or two days after the first, resulted in a slower rate of recovery. However, if recovery time is calculated from 0 days, that is, from the time of the first dose, the time of recovery is within 18 to 20 days for any of the doses used. Thus even though the repeat doses were given at different intervals it made little difference in total time needed for recovery.

Table I is better understood if some cytological features are described. It has been stated that when prespermatogonia are not obvious they are listed as 0 although it is understood that they are always present. When prespermatogonia are obvious the incidence of appearance of these cells in tubules varies from 4 to 19 per cent. Any incidence higher than this highest figure is considered to indicate that a period of repopulation or recovery is in progress.

Recovery is defined as that condition where as many tubules show the presence of gonial cells as in unradiated control animals. This is not total recovery, however. Actually total recovery would be achieved only when all the tubules of a testis have the usual complement of germ cells. The recovery is real however, if not total, for the presence of prespermatogonia indicates that total recovery is only a matter of continuing development. The prespermatogonia in some areas of the testis prior to the recovery interval are actively undergoing change into spermatogonia. In some cases, some spermatogonia have developed into early spermatocytes. It is not the intent of Table I to indicate that all sections of the testis are in the same stage of development. After 20 days spermatogonia would be so frequent that prespermatogonia would become less obvious and subsequent restoration would be from spermatogonial activity as in control animals.

SUMMARY

The testes of 68 partially shielded animals were irradiated with non-lethal acute doses of 138r and 300r. In another series, 144 animals were given a dose of 138r followed by a repeat dose of 138r (totalling 276r) at the selected intervals of 1, 2, 4, or 8 days.

Animals were killed at intervals post-radiation and the germinal epithelium examined microscopically. Effect of irradiation was followed on spermatogonia and prespermatogonia.

By noting the per cent of tubules showing either of the single-type cells on the listed days post-radiation it is possible to indicate graphically the effects of a single dose or of repeat doses of x-irradiation.

Three factors were considered:

1) The time necessary for depopulation of spermatogonia; 2) The time period of the effect of radiation as indicated by the first evidence of restoration by prespermatogonia; and 3) The time necessary for recovery determined by the return to near normal frequency of spermatogonia.

Depopulation of the spermatogonia occurred in 3–4 days after an acute dose of 138r.

The time period of the effect of the irradiation was found to be 5–8 days after an acute dose of 300r. Repeat doses of 138r at intervals of 1, 2, 4, or 8 days (totalling 276r) gave a similar interval of effect after the last dose.

Recovery of normal incidence of appearance of spermatogonia is delayed following repeat doses when contrasted with an acute dose of 300r. It makes little difference, however, if the repeat dose is given 1, 2, 4, or 8 days after the first dose.

This study of effect of irradiation on a single-type cell confirms clinical observations that recovery is delayed after a repeat dose.

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ENZYMATIC STUDIES OF CHANGES IN ADENOSINETRIPHOSPHATE DURING CONTRACTION OF ISOLATED SKELETAL MYOFIBRILS[1]

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The enzymes in muscle and other tissues which act on ATP[2] and its related compounds appear to be necessary for the release of chemical energy for many types of cellular function. Apparently, the high energy phosphate bond of ATP is the principal source of this energy. In muscle this energy is utilized in the performance of mechanical work. The present study was concerned with the reaction between ATP and structurally intact isolated skeletal myofibrils.

Schick and Hass (10, 11) described a method for the isolation of skeletal and cardiac myofibrils from rabbit muscle by tryptic digestion at 0°C and have studied the solubility properties of these fibrils. Myofibrils were also isolated by mechanical methods using the high shearing forces of a colloid mill. For skeletal myofibrils the latter method proved more satisfactory than the enzymatic method, since large quantities of myofibrils could be easily obtained and the removal of the Z band and distortion of the I band noted in the preparations treated with trypsin was avoided. These intracellular structures, when they were separated from the cell, still retained the property of contracting instantaneously on the addition of ATP.

Microscopically these myofibrils showed the major cross striations, the A and I bands (Fig. 1). Observation during the addition of ATP revealed the myofibrils to contract into small round or oval masses (Fig. 2). At room temperature contraction occurred in less than one second. The rate of contraction was reduced by lowering the temperature. The degree of contraction was found to be related to the amount of ATP added.

The electron microscope was used to study the structure of these myofibrils, including the pronounced changes associated with contraction induced by

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[2] Abbreviations used: ATP—adenosinetriphosphate; ADP -adenosinediphosphate; AMP—adenylic acid; ATPase—adenosinetriphosphatase.

ATP (1). These studies suggested the possibility of an acquisition by the myofibrils of some substance from the supernatant fluid during contraction. Fig. 3 is an electron micrograph showing the structure of myofibrils isolated with the colloid mill in the uncontracted state. Fig. 4 is an electron micrograph of a myofibril contracted with ATP. The sarcomere length is one-half that of the sarcomere length of the average uncontracted myofibril. The shift in the structural pattern with the formation of a dense contraction band is shown. These changes are similar to those observed by classical histological technique (4).

Detailed investigations (8, 9) have been made of the enzymes present in the myofibril which act on ATP. Emphasis was not placed on the occurrence or absence of contraction when the ATP was added under the conditions of the experiments. Since contraction of the myofibrils is almost instantaneous at room temperature, it is doubtful that phosphorus liberated either minutes or hours after the addition of ATP has significance for the actual contractile event.

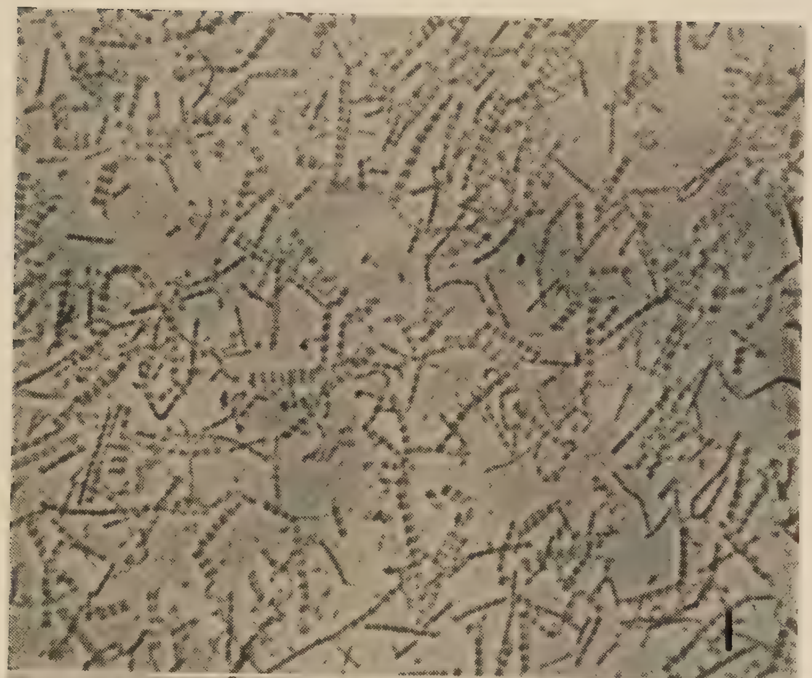
In the present study the chemical reaction between ATP and myofibrils which were purified and washed until the supernatant contained no detectable protein and enzymes was investigated. The amount of ATP necessary for contraction was added to the myofibril suspension. Excess amounts of ATP were avoided. Contracted myofibrils were centrifuged and the supernatant removed for analysis within less than five minutes following the addition of the ATP.

The three compounds, ATP, ADP and AMP, have similar absorption curves in the ultraviolet region (6). Measurement of the ultraviolet absorption of this supernatant compared with the supernatant of controls was used to detect any persistent binding or combination of these nucleotides with the myofibrils during or after contraction. The conditions of this experiment were modified by varying the pH and the amount of ATP added to a given amount of myofibril suspension.

The number of phosphate groups removed from ATP while in contact with the myofibrils and, indirectly, the enzymes present within the myofibrils which act upon ATP were determined by Kalckar's enzymatic and spectrophotometric method for the analysis of a mixture of ATP, ADP and AMP (6).

METHODS

Isolation and purification of myofibrils. Myofibrils were separated from 50 g of rabbit skeletal muscle placed in 150 ml of 0.15 M KCl solution in an Eppenbach Colloid Mill, Type QV-7-1. The anterior thigh muscles gave the most satisfactory



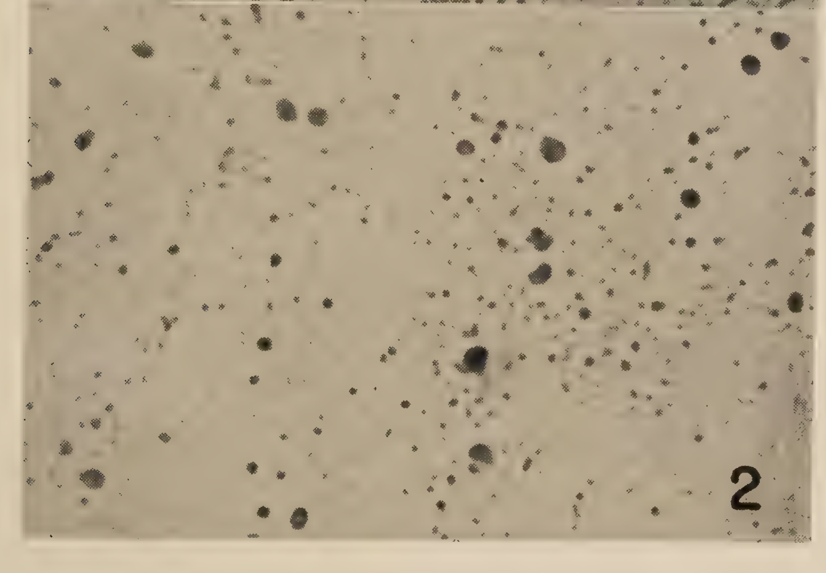
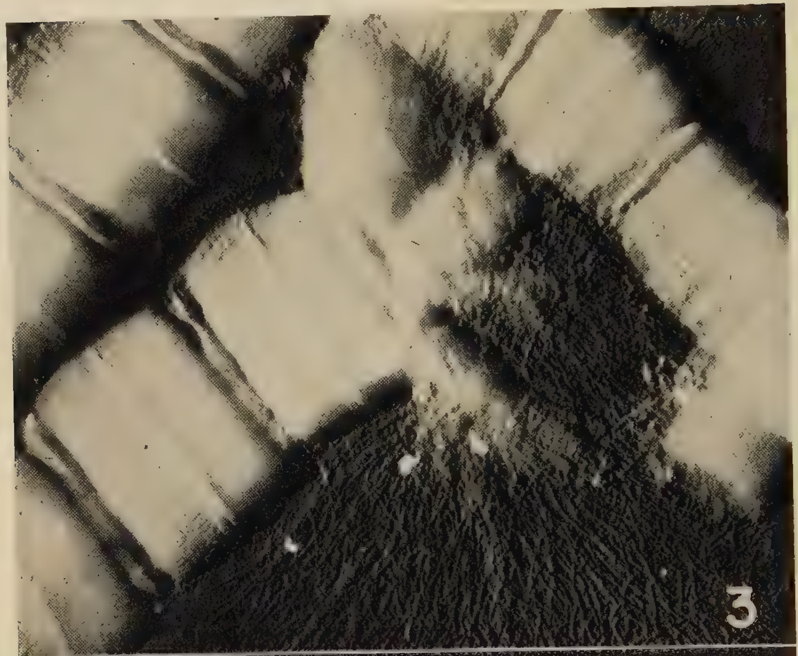


Fig. 1. Photomicrograph of mill-isolated rabbit skeletal myofibrils in the uncontracted state. × 900.

Fig. 2. Photomicrograph of similar myofibrils following the addition of ATP, showing the round or oval masses resulting from contraction. × 1100.



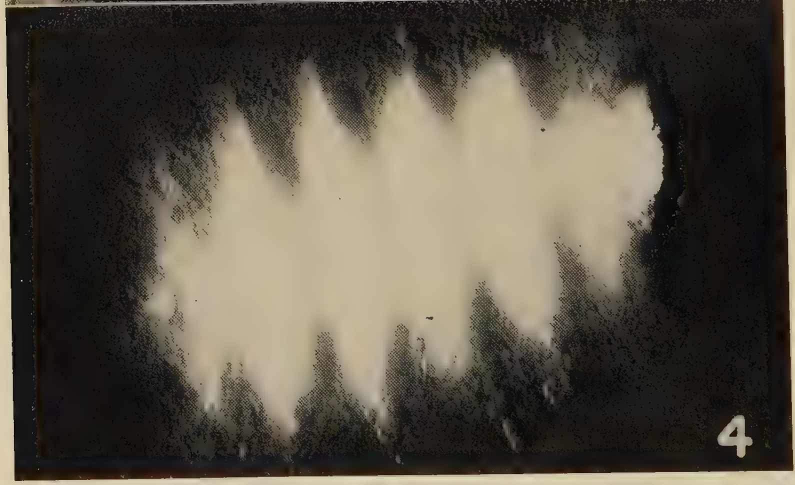


Fig. 3. Electron micrograph of mill-isolated rabbit skeletal myofibrils fixed with buffered formalin, dried and shadowed with chromium. The characteristic cross striations are shown. The ruptured end of one of the fibrils shows the filaments of which they are composed. × 14,000.

Fig. 4. Electron micrograph of similar myofibril which was contracted with ATP, fixed with buffered formalin, dried and shadowed with chromium. The sarcomere length is 50 per cent of that of the uncontracted myofibrils. The dense elevated contraction band is shown. × 14,000.

results. This mill was equipped with a water-cooled jacket and a recirculating device. The distance between the rotor and stator could be altered and was calibrated in arbitrary units. The optimal distance and speed were determined by trial and error. The above proportions of muscle and solution gave a very viscous, thick suspension. The mill was run for a period of 15 seconds with the recirculating device open. A drop of material was examined microscopically to determine the degree of rupture of the cells and the number of free myofibrils in the suspension. Milling was continued until an estimated 50–75 per cent of the muscle fibers had been completely broken down into free myofibrils. This was usually accomplished in less than one minute of operation. At this point the suspension contained enormous numbers of free myofibrils, occasional small clumps of fibrils, few intact nuclei and many very fine granules. All subsequent procedures were carried out in a cold room at 0°C.

The myofibrils were purified by differential centrifugation in this same solution and subsequently washed repeatedly in a potassium phosphate-citric acid buffer solution (pH 8.0, μ 0.15). (The alkaline solution was found suitable for removal of all myokinase activity.) The supernatants were saved and the protein content estimated by the Folin-Ciocalteau phenol reagent (3). The washing was continued until the concentration of protein in the supernatant was too low to be determined by this method, indicating a concentration of less than 20 micrograms per ml. The ultraviolet absorption curve of this supernatant was also too low to be determined with a Beckman Quartz Spectrophotometer, Model DU.

The fibrils were then suspended in a solution containing nine parts of 0.15 M KCl, one part of succinate buffer solution (pH 6.1, 0.16 M), and 0.5 g $MgCl_2$ per liter, and were washed three times in this solution. These myofibrils retained the property of contracting instantaneously on the addition of ATP at room temperature.

Recovery of nucleotides. 0.5 ml (approximately 0.050 g dry weight) of centrifuged, packed myofibrils were suspended in 10 ml of solution containing 9 parts of 0.15 M KCl, one part of succinate buffer solution (pH 6.1, 0.16 M) and 0.5 g $MgCl_2$ per liter. The amount of the sodium salt of ATP necessary to insure complete contraction of this quantity of myofibrils was determined by microscopic observation and found to be approximately 0.003 g. The myofibrils were contracted by addition of ATP and then immediately centrifuged. The supernatant was collected and filtered. After appropriate dilution the ultraviolet absorption curve of this filtrate was determined and compared with that of three similarly treated controls. The first control consisted of an equal amount of ATP in buffer solution. For the second control, this amount of ATP was added to the supernatant removed from uncontracted myofibrils. 0.004 molar copper sulfate inhibited contraction. The third control consisted of the supernatant of myofibrils to which both copper sulfate and ATP were added.

These experimental conditions were modified in two ways. Progressively smaller amounts of ATP were used, down to one-tenth (0.0003 g) of the amount used above. This quantity of ATP caused no contraction that could be detected by microscopic observation. The ultraviolet absorption curves for the supernatant of the myofibrils to which this amount of ATP was added were compared with those of controls in the manner already described.

The experiment also was modified by keeping the amount of ATP constant but using different buffer solutions with pH values of 6.0, 6.6, 7.0 and 7.6. These consisted of nine parts of KCl (0.15 M) and one part of the appropriate potassium phosphate-citric acid buffer solution. The purified myofibrils were washed four times in one of these solutions. ATP was added, and the ultraviolet absorption curves of the supernatant and controls were determined.

Chemical changes in the nucleotide. Kalckar's enzymatic method (6) for the quantitative determination of a mixture of ATP, ADP and AMP was used. This method depends on the shift in the ultraviolet absorption curve when AMP is enzymatically deaminated to form inosinic acid. Of the three compounds, only AMP is deaminated by this enzyme (Schmidt's adenylic acid deaminase). Comparison of the ultraviolet absorption curves for AMP and inosinic acid shows the greatest difference at 265 millimicrons. When adenylic acid deaminase is added to AMP, the decrease in absorption at 265 millimicrons is proportional to the amount converted to inosinic acid by deamination. Myokinase converts two molecules of ADP to one of AMP and one of ATP by shifting a phosphate group from one ADP molecule to the other. A pyrophosphatase found in potato splits off one phosphate group from ADP and two phosphate groups from ATP, thus converting both compounds to AMP.

The analysis of a mixture of the three compounds (ATP, ADP and AMP) was carried out by first adding only adenylic acid deaminase. This was prepared according to the methods of Schmidt and Kalckar (12, 7). The decrease in ultraviolet absorption at 265 millimicrons was proportional to the amount of AMP. To a second tube, both myokinase, prepared according to the method of Colowick and Kalckar (2), and deaminase were added. The decrease in absorption was proportional to the amount of AMP, plus one-half the amount of ADP. In a third tube, potato adenylpyrophosphatase prepared according to Kalckar (7) was added along with the myokinase and the deaminase. The drop in absorption was proportional to the amount of AMP, plus ADP, plus ATP. Each of the enzymes prepared by these methods was tested for activity with the three compounds, ATP, ADP and AMP. Those preparations which were active with only the specified compounds were used in the analysis.

This method of analysis was applied to the supernatant of the contracted myofibrils and control solutions. These consisted of ATP in buffer solution, ATP added to the supernatant of uncontracted myofibrils, and ATP added to the supernatant of fibrils already contracted with ATP. The copper sulfate control used in the recovery experiments wherein contraction of the myofibrils was inhibited could not be used, since the analytical method depended on enzymatic reactions inhibited by the copper ion.

The concentration of protein in the supernatant of contracted myofibrils and appropriate controls was determined by the Folin-Ciocalteau phenol reagent (3).

RESULTS

The recovery experiments designed to indicate any binding of nucleotide during or after contraction all gave similar results. Since ATP, ADP and AMP all have similar absorption curves in the ultraviolet with a maximum at 260 millimicrons, the total amount of nucleotide was measured by the absorption at this wave length. Measurements were made on the supernatant of myofibrils contracted with ATP and compared with the controls; namely, ATP in buffer solution, ATP added to the supernatant removed from uncontracted myofibrils and ATP added to myofibrils, the contraction of which was inhibited by copper sulfate. In none of these experiments was there any evidence of permanent, large-scale binding of the nucleotides to the myofibrils during or after the process of contraction. The supernatant of the contracted myofibrils contained 95 to 99 per cent of the total amount of nucleotide present in all of the controls.

Microscopic observations indicated that the degree of contraction of the myofibrils was dependent on the amount of ATP added. The addition of a small amount of this compound was accompanied by slight contraction. Further contraction could be obtained by adding more ATP. That binding of the nucleotide might be obscured by the use of a large excess of ATP was considered. The use of progressively smaller concentrations of ATP down to an amount which failed to cause microscopically detectable contraction would have shown a decreasing nucleotide recovery in the supernatant if there were any significant binding. In none of these experiments was the nucleotide in the supernatant of the contracted myofibrils less than 95 per cent of that of the controls.

The use of different hydrogen ion concentrations did not affect the recovery of the nucleotide. When the myofibrils were first washed and then suspended in buffer solutions, the pH of which varied between 6.0 and 7.6, more than 95 per cent of the total nucleotide was always recovered in the supernatant.

The results of a single experiment illustrating the changes in nucleotide composition associated with contraction are shown in Table I. The ATP stock solution showed marked variation in its composition from time to time. Table II shows the results from five consecutive experiments, in which the ATP varied from 57–75 per cent of the total nucleotide in the stock solution. In order to compare and average the results of the different experiments, the amount of ATP in the stock solution for each experiment was considered 100 per cent. In the supernatant of the contracted myofibrils and in the controls, the net change in the amount of ATP, ADP and AMP was expressed as a per cent of the amount of ATP in that stock solution. Since the figures indicate the net change in composition, the values for the ATP in buffer solution or base-line values are zero.

The absence of any significant enzymatic splitting of the ATP by the supernatant of the uncontracted myofibrils is shown (3 of Tables I and II). In both the single experiment and in the average of the five experiments the composition of the ATP in buffer solution was not significantly altered by the addition of the same amount of ATP to the supernatant first removed from the uncontracted myofibrils. (Compare 1 and 3 of Tables I and II). This indicated that the suspending medium was free of enzymes acting on the ATP and that any change in the nucleotide composition of the supernatant of the contracted myofibrils must have resulted from the action of the myofibrils or an intimately associated enzyme.

TABLE I

Analysis of nucleotide composition of supernatant of myofibrils contracted with ATP and controls; single experiment.

	Percentage composition			Per cent Accounted
	ATP	ADP	AMP	
1. ATP in buffer solution	75	7	2	84
2. Supernatant of myofibrils contracted with ATP.	0	86	4	90
3. Supernatant of uncontracted myofibrils with ATP added in absence of myofibrils	73	3	6	82
4. Supernatant of myofibrils contracted with ATP to which additional ATP was added	42	34	5	81
5 Average of 1 and 2	38	46	3	—

In contrast to the absence of enzymatic splitting of ATP by the suspending medium, the supernatant of myofibrils contracted with ATP (2 of Tables I and II) showed a 97 per cent average decrease in the amount of ATP. The recovery experiments above showed that, of the added nucleotide, at least 95 per cent was recovered in the supernatant, so that this decrease in the amount of ATP was accompanied by a nearly corresponding increase in the amount of ADP. These results indicated that the myofibrils contained an ATPase capable of removing the terminal phosphate group from ATP. The absence of myokinase in the system was also shown, since, were it present, the cycle theoretically would have continued until at least 30 per cent of the ADP had been converted to AMP (5).

The possibility was considered that during or after contraction there might have been a release of enzyme (ATPase) from the myofibril into the suspending medium. In order to test this possibility, the supernatant of the myofibrils contracted with ATP which would have contained this enzyme was tested for enzymatic activity. The nucleotide composition of this supernatant was analyzed and contained almost no ATP and large amounts of ADP (2 of Tables I and II). Equal volumes of this supernatant of contracted myofibrils and the stock ATP in buffer solution, the nucleotide composition of which was also known, were combined. The total nucleotide concentration of each of the solutions was the same. This combined solution was then analyzed for ATP, ADP and AMP.

If active ATPase were present in the supernatant, the added ATP would have been degraded to ADP and the results would have been similar to those for the supernatant of the myofibrils contracted with ATP (2 in Tables I

TABLE II

Net change in nucleotide composition expressed as a per cent of the concentration of ATP in buffer solution in five consecutive experiments.

		Percentage change in composition		
		ATP	ADP	AMP
1. ATP in buffer solution		0	0	0
2. Supernatant of myofibrils contracted with ATP	Exp. a	−100	+102	0
	Exp. b	−100	+ 97	−14
	Exp. c	− 88	+ 75	− 6
	Exp. d	−100	+105	+ 3
	Exp. e	− 96	+ 78	−15
	Average	− 97	+ 91	− 6
3. Supernatant of uncontracted myofibrils with ATP added in absence of myofibrils	Exp. a	− 7	+ 9	+ 9
	Exp. b	+ 7	− 2	−14
	Exp. c	+ 9	− 19	+16
	Exp. d	− 3	− 5	+ 5
	Exp. e	− 13	+ 3	−12
	Average	− 1	− 3	+ 1
4. Supernatant of myofibrils contracted with ATP to which additional ATP was added	Exp. a	− 33	+ 47	+18
	Exp. b	− 68	+ 54	− 7
	Exp. c	− 41	+ 41	− 6
	Exp. d	− 44	+ 36	+ 4
	Exp. e	− 62	+ 54	−22
	Average	− 50	+ 46	− 3
5. Average of 1 and 2		− 49	+ 46	− 3

and II). If no additional enzymatic reactions occurred, the nucleotide composition would theoretically equal the average of the two combined solutions (5 of Tables I and II). The results (4 of Tables I and II) show good approximation to the results expected if there were no loss of enzyme into the suspending medium during contraction and, therefore, no degradation of the additional ATP.

Any loss of protein from the myofibril during the contraction process would appear in the supernatant of the contracted myofibril. The determined protein concentration of this supernatant did not differ significantly from that of the controls.

DISCUSSION

The myofibril, which still contracts on the addition of ATP after isolation from the cell and purification, is a convenient system in which to study the theoretical reactions which convert the chemical energy of this compound into cellular function. The degree of contraction was dependent on the amount of ATP added. This suggested that the energy of the compound was exhausted, since additional ATP caused further contraction.

These studies showed no evidence of any enzyme in the suspending medium. During contraction, the terminal phosphate group was split from ATP, resulting in ADP which was almost quantitatively recovered in the supernatant of the myofibrils after contraction. All evidence indicated that the enzyme responsible for this degradation of ATP was a portion of the structure of the myofibril. The speed of contraction, which was almost instantaneous at room temperature and required only a few seconds at 8°C, is difficult to explain on the basis of ordinary enzyme kinetics. Although ATP splitting was indicated in all of the experiments, it has not been shown that this is the only reaction necessary for contraction. The possibility remains that this splitting occurs independent of contraction.

The nearly complete recovery in the supernatant of the added ATP as ADP does not exclude the fact that the compound must have combined with the myofibril either before or during contraction. The time necessary for centrifugation of the contracted myofibrils and removal of the supernatant may have been sufficient to allow the ADP to dissociate from the myofibril. It is also possible that the methods used were not of sufficient sensitivity to detect the permanent binding of a small but significant portion of the added ATP. These studies suggest that this would have been less than 5 per cent of the total added nucleotide. When progressively smaller amounts of ATP, down to an amount which failed to cause microscopically detectable contraction, were added to the myofibrils, 95 per cent or more was always recovered in the supernatant as ADP. In this experiment an excess of ATP was not used, but there was no evidence of a decreased recovery of nucleotide.

The purification methods resulted in the removal of myokinase activity from the myofibril. This enzyme converts two molecules of ADP to one molecule of ATP and one molecule of AMP. This reaction reaches an equilibrium in which 60 per cent of the ADP is converted into these two compounds. The reaction goes to completion in the presence of adenylic acid deaminase (6). If myokinase had been present in the myofibril, at least 30 per cent of the added ATP would have been recovered as AMP and it is probable that the reaction would have gone to completion since the myofibrils were shown to contain ATPase and were never completely free of deaminase activity. The results indicated no significant increase in the amount of AMP. It is unlikely that this enzyme was present in the myofibrils and inactive because of the experimental conditions, since it was added to the medium in the analytical procedure and was active.

SUMMARY

Skeletal myofibrils, isolated from rabbit thigh muscle with a colloid mill in 0.15 M KCl, were purified by differential centrifugation at 0°C and washed repeatedly with potassium phosphate - citric acid buffer solution (pH 8, μ 0.154) until the protein concentration in the supernatant was less than 20 micrograms per ml. The fibrils were then washed and suspended in a solution containing 9 parts of 0.15 M KCl, one part of 0.16 M succinate buffer (pH 6.1) and 0.5 g $MgCl_2$ per liter. These myofibrils contracted instantaneously on the addition of ATP. The degree of contraction was related to the amount of ATP added.

The amount of nucleotide recovered in the supernatant of contracted myofibrils was determined by the ultraviolet absorption at 260 millimicrons and compared with controls. These conditions were modified by varying the pH of the suspending medium between 6.0 and 7.6 and by adding decreasing amounts of ATP down to an amount which failed to cause microscopically detectable contraction. Analyses using Kalckar's enzymatic and spectrophotometric methods for ATP, ADP and AMP were made on the ATP stock solution, the supernatant of myofibrils contracted with ATP, the supernatant of uncontracted myofibrils with ATP added in the absence of myofibrils, and the supernatant of ATP-contracted myofibrils to which additional ATP was added.

There was no indication of permanent, large-scale binding of ATP or ADP to the contracted myofibril, since in all experiments 95 per cent or more of the added nucleotide was recovered in the supernatant. The structurally intact, purified myofibrils were free of myokinase activity. The added ATP was converted to ADP which could be recovered almost quantitatively in the supernatant. The ATPase which catalyzed this reaction was a part of the structure of the myofibril, since the supernatant of uncontracted myofibrils had no ATPase activity. There was no passage of ATPase or detectable amounts of protein into the suspending medium during contraction.

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THE EFFECTS OF OSMIUM TETROXIDE FIXATION ON THE STRUCTURE OF MYELIN IN SCIATIC NERVE

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Osmium tetroxide has been used extensively as a fixative in the preparation of biological material for electron microscopy, but little is known of its specific action, particularly on the ultrastructure, and consequently a detailed interpretation of the electron micrographs in terms of the normal structure is usually not possible. Previous surveys (1, 12, 13) of the application of this method of fixation have emphasized the need for more specific information concerning its effects.

Interaction with osmium tetroxide appears to be most marked in the case of systems containing lipids, and the highly ordered structure of nerve myelin seemed to offer a useful system for a more detailed study of its effects on this type of material.

The structure of myelin has been studied extensively by polarized light (15), X-ray diffraction (15–17, 2–4, 9, 10), and electron microscopy (5–8, 14), and in the latter work the myelin was usually fixed with osmium tetroxide during preparation. In the electron micrographs of thin sections of OsO_4-fixed sciatic nerve obtained by Fernández-Morán (5–8) using the freeze-sectioning technique, and by Sjöstrand (18) using an embedding technique, a structure consisting of alternating light and dense layers was clearly shown. These layers appear to be wrapped concentrically around the axis cylinder. In addition to this dense osmium deposition in broad bands, the electron micrographs obtained by Sjöstrand also showed a thin line of dense deposition running approximately through the middle of the layer which was otherwise relatively lightly stained. The general picture obtained from electron micrographs of thin sections of OsO_4-fixed sciatic nerve is thus very similar to that deduced for the myelin sheath of normal sciatic nerve from polarized light and X-ray diffraction data.

This paper reports a number of observations on the effects of OsO_4-fixation on the diffraction patterns of nerve which help to link up the X-ray diffraction and electron microscope investigations.

EXPERIMENTAL

Materials and Methods. Sciatic nerve preparations from frog and rat were treated with 1 per cent solutions of OsO_4 in M/15 phosphate buffers at pHs between 6.5 and 7.5. Both normal fresh material and specimens already modified by freezing or drying were treated in this way. In addition, a number of specimens were first fixed with formalin and then treated with OsO_4 solution. The effects of OsO_4 on a number of isolated lipids including specimens of synthetic phosphatidyl ethanolamines (dipalmitoyl, dimyristoyl, erucoyl-stearoyl, and linoleoyl-palmitoyl) were also studied.

After fixation, the specimens were washed, and then sealed up either in Ringer solution or in distilled water in thin-walled glass capillary tubes.

The X-ray diffraction camera (11) and observation cells (2, 3) have been described. Nickel-filtered Cu Kα radiation was used throughout.

RESULTS

In unbuffered OsO_4 solutions, the low angle diffraction pattern of sciatic nerve was almost completely destroyed. Well-defined diffraction patterns were obtained from specimens after fixation in 1 per cent OsO_4 solution buffered to a pH of 6.5 to 7.5.

When normal frog sciatic nerve was placed in the buffered OsO_4 solution, the low angle diffraction pattern was quickly modified. The most striking change was a marked increase in the intensity of the diffraction in the region of 150 to 170 Å which was initially extremely weak. This was accompanied by a much slower general reduction of spacings, the highest spacing being reduced to a steady value of about 148 Å after about two hours. The modified diffraction pattern is shown in Fig. 1 a (upper pattern).

The diffraction patterns of specimens in Ringer solution and those in distilled water showed no appreciable differences.

The wide angle pattern of OsO_4-fixed sciatic nerve showed a diffuse ring at 4.7 Å. The 15 Å equatorial spots of the normal nerve pattern were not observed.

When the fixed specimen was subsequently dried, the low angle diffraction pattern (Fig. 1 a, lower pattern) showed two bands at 116 Å and 58 Å, the former being the more intense but often only slightly so. The 4.7 Å ring was again broad and diffuse. Specimens which were re-immersed in Ringer solution showed an appreciable expansion of structure and a tendency for the diffraction pattern to resume its original (freshly fixed) appearance.

The observations made with rat sciatic nerve were very similar in character, but the spacings recorded were in general a little higher than in the case of frog material. An additional difference was that whereas the intensities of the diffraction bands in the low angle pattern of the OsO_4-fixed frog sciatic showed a regular decrease with increasing order, in the pattern of the corresponding rat specimen the third order diffraction appeared unusually intense.

Effects of temperature and of lipid solvents on frog sciatic nerve after OsO_4 fixation. In the frozen state, OsO_4-fixed frog sciatic nerve showed two intense low angle diffractions at about 128 Å and 64 Å. On thawing, the diffraction pattern resumed its original form. When subsequently dried, the specimen gave a diffraction pattern identical with that of the specimen dried without freezing.

When OsO_4-fixed nerve was kept in boiling water for about thirty minutes and then allowed to cool, the diffraction pattern subsequently obtained showed spacings identical with those of the freshly fixed specimen, but the orientation was relatively poor (Fig. 1 b, upper pattern). After drying, the diffraction spacings were again identical with those of the corresponding untreated specimen, but the orientation was poor (Fig. 1 b, lower pattern).

Treatment of OsO_4-fixed specimens with hot alcohol for short periods also failed to produce any appreciable changes in diffraction spacings.

In the case of specimens which had been inadequately fixed (e.g. for too short a time), some degree of modification similar to that observed with unfixed nerve could be produced by these treatments.

Effects of OsO_4 on modified nerve. In several experiments, the fresh nerve was subjected to freezing and thawing, or was first dried, before treating with the buffered OsO_4 solution. When examined in the wet state after fixation, such specimens gave an X-ray diffraction pattern showing a heavy background scatter with one or two faint diffraction bands superimposed. The positions of these bands varied somewhat with different types of specimens but in general they were in the ranges 50 to 60 Å and 70 to 80 Å. No well-defined bands were observed at higher spacings, but there was a heavy general scatter in the region above about 120 Å. After drying, the diffraction bands became clearer, but the intensity and definition was not comparable with that of the OsO_4-fixed normal specimen.

Fresh nerve specimens which had been placed in 4 per cent formol saline for some hours showed two intense low angle diffractions at about 86 Å and 57 Å. This pattern is shown in Fig. 1 c (top) and that of the corresponding dried specimen in the lower pattern. When treatment with OsO_4 solution followed the formalin fixation, subsequent X-ray diffraction patterns showed a heavy diffuse scatter at low angles and a faint diffraction band at about 50 Å. After drying, two faint bands were observed in the 50 to 60 Å region.

Effects of OsO_4 on isolated lipids. When a rolled specimen of crude brain lecithin which in the normal state gave a sharp diffraction at about 60 Å, was immersed in OsO_4 solution for a few hours and then examined, only a heavy diffuse low angle scatter was observed. After drying, the 60 Å band reappeared, apparently as intense and well-defined as in the untreated state. The heavy general scatter persisted. After fixation, the lipid was much less readily soluble in lipid solvents (e.g. chloroform) than the unfixed material. In the case of the synthetic phosphatidyl ethanolamines, the specimen having a monoethenoid fatty chain reacted with OsO_4 much more quickly than those with fully saturated chains. However, the latter did eventually blacken in the OsO_4 solution. These pure specimens did give a diffraction pattern in the wet state after fixation, and the principal long spacing observed was only slightly higher than that of the untreated dry lipid. After drying, the

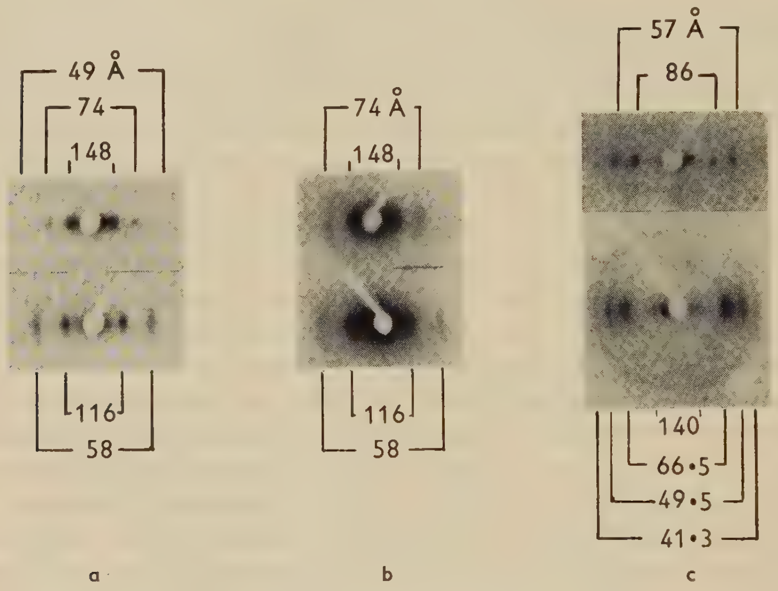

Fig. 1 a. Low angle diffraction patterns of OsO_4-fixed sciatic nerve. Upper pattern—moist nerve. Lower pattern—dried nerve.

Fig. 1 b. Corresponding patterns of specimen which has been heated in boiling water for thirty minutes after fixation.

Fig. 1 c. Low angle diffraction patterns of formalin-fixed sciatic nerve. Upper pattern—moist nerve. Lower pattern—dried nerve.

spacing did not differ appreciably from that of the unfixed material, but in the case of the erucoyl-stearoyl cephalin the second order diffraction of the OsO_4-fixed specimen was certainly more intense. In the case of the linoleoyl-palmitoyl specimen which was probably already slightly oxidized, the reaction with OsO_4 was very rapid, but the moist specimen gave no diffraction pattern. Even in the dried state, the pattern was faint and ill-defined.

DISCUSSION

Osmium tetroxide in buffered solutions at about pH 7 brings about striking changes in the low angle diffraction pattern of sciatic nerve. There is a general increase in intensity of diffraction, and a marked change in the relative intensities of the bands. The orientation of the pattern is preserved, and all the diffraction bands can be related to one radially-orientated structural unit. The intensification of diffraction is probably the direct contribution of the osmium deposited in the structure, and the fact that the definition of the pattern is preserved indicates a deposition at specific points which repeat in a regular fashion in the radial direction. The repetition of these points emphasizes the full unit cell dimension, and the tendency for halving which is so marked in the pattern of fresh myelin is no longer observed after osmium tetroxide fixation. The chemical action of OsO_4 is probably one of oxidation, and there would appear to be only a limited number of groups likely to be present in myelin which are capable of reacting with this reagent. It has been shown that OsO_4 will readily oxidise the olefinic linkages in unsaturated lipids. Saturated glyceryl esters and phospholipids which have fully saturated hydrocarbon chains blacken only very slowly in OsO_4 solutions. Cholesterol has one unsaturated bond in the ring structure, and reaction with OsO_4 would seem to be possible though crystalline cholesterol does not appear to blacken readily in OsO_4 solution. Reaction of OsO_4 with protein molecules has been demonstrated, and it has been suggested that one of the principal sites of interaction is at the double bonds of tryptophane side chains. This reaction would appear to be relatively slow.

The fatty acids found in the myelin lipids are largely saturated acids and monoethenoid fatty acids, and it is possible that the latter provide the main interactions with OsO_4. For a satisfactory interpretation of the diffraction pattern, the double bonds must be grouped at well-defined levels in the unit cell and show some asymmetry with respect to the long axis. From previous work it has been suggested that as far as the general arrangement of lipid and protein layers is concerned, the two halves of the unit cell are very similar. In the normal structure the unsaturated double bonds probably have relatively little effect on the diffraction pattern, but as soon as they become associated with the heavy osmium atom they will become the dominating factor in diffraction. Perhaps the simplest explanation would be provided by the case in which the amount or the type of unsaturated lipid varied appreciably in alternate layers, and such an arrangement would also provide possible interpretations of a number of additional facts observed in these experiments. It is observed that the second order diffraction becomes much more intense in the dried specimen. This would suggest that in the dried specimen strongly diffracting groups repeat at the half cell distance which were not so placed in the moist specimen. These may be groups with osmium attached, or possibly groups which were responsible for the intensity variations in the original fresh nerve and whose position in the unit cell was changed during fixation. These changes are accompanied by reductions in the length of the unit cell amounting to about 22 Å on fixation and a further 32 Å on subsequent drying to constant weight, though dehydration by freezing effects only a 20 Å reduction in spacing. The addition of an OsO_4 group at the ethylenic double bond of the lipid chains will almost certainly affect the packing of such chains and may lead to tilting, with consequent contraction of the layers. If the lipids of alternate layers differ, it is possible that layers contract by different degrees so that in the moist specimen no important groups repeat at the half cell distance. The additional contraction associated with drying may again bring groups into positions where they can reinforce the second order diffraction. Possible interaction of OsO_4 with cholesterol or with the protein might also be fitted into such a scheme but definite conclusions cannot be drawn in the absence of precise knowledge of the interactions, and this speculation can only be regarded as a general illustration of the implications of the X-ray diffraction data.

The properties of the structure are greatly affected by this fixation. Thus, on drying, there is no evidence for the separation of free lipid components such as were shown to be present in normal dried nerve. The so-called labile lipid component is presumably bound more firmly into the structure by the action of OsO_4. This has a marked effect on the action of solvents on fixed material. It has been shown previously that ether and acetone will extract the free lipid components which separate from the main structure during the initial drying, but will not readily remove the lipid which remains associated with the protein component. These solvents have no effect on the OsO_4-fixed material. Even alcohol, which is capable of breaking down all the associations in the normal structure so that most of the lipid can be extracted, is ineffective after OsO_4-fixation. In most cases the process of extraction appears to consist of a breaking down of the complex structure, and then a solvation of the relatively simple components. The effect of OsO_4 may be to bind the lipid more firmly to the protein, or possibly to bind the lipid molecules or complexes more tightly together so that the existing strong links with the protein hold the whole structure. Even in purely lipid systems, OsO_4 appears to lower the solubility to a very marked degree. The effects of freezing and heating on normal nerve have been associated with a denaturation of the protein component with consequent breaking of links between lipid and protein, this in turn resulting in a breakdown of some of the lipid complexes and expansion of the structure. It is probably safe to assume that after OsO_4-fixation, the protein is already denatured, but the structure has not expanded. This would imply that the complex lipid form still exists, and the fact that by heating in boiling water disorientation can be produced without any apparent breakdown of structure suggests that the lipid is firmly bound to the protein.

It is of interest to note that although the structure probably remains intact on drying, orders of diffraction higher than the second are no longer observed. A similar effect was observed on drying normal nerve, only the first and second order diffractions of the "residual complex" being recorded, and this is now shown to be due not so much to the separation of some of the lipid components as to a shift of strongly diffracting groups away from positions in which they were able to reinforce these higher orders of diffraction. Similar changes have been noted in other modification experiments, and an attempt is now being made to correlate all these intensity variations observed in different experiments in the hope that they will yield more detailed information on the structure of myelin. A probable feature of the fresh nerve structure is the precise alignment of reactive groups (double bonds) in the planes perpendicular to the radial direction, and this may be the essential difference between normal nerve and modified nerve which accounts for the different effects of OsO_4 on the systems. The modified nerve specimens certainly react with OsO_4, but no diffraction pattern is observed. The suggestion is that in such specimens the osmium deposits must be distributed somewhat at random in the radial direction, and the modifications can therefore probably be expressed in terms of a change from a precise distribution of reactive groups to a random one. This can probably be reconciled with the suggestion from earlier work of a change from a highly organized lipoprotein system to a mixed lipid-lipoprotein system.

The effect of OsO_4 on formalin-treated nerve is similar to that with nerve initially modified by such treatment as freezing, and furthermore the diffraction pattern of the formalin-treated specimen shows intensification of the band at about 58 Å which in other experiments has been associated with the separation from the main structure of a labile lipid component. This might be taken to suggest that formalin does not preserve the intact structure.

Unbuffered OsO_4 solution also destroys the diffraction pattern, and it seems likely that the conditions are such as to produce a modification of structure before fixation can take place.

It is of interest to try to correlate these general conclusions concerning the reaction of OsO_4 with fresh nerve myelin with the electron micrographs of OsO_4-fixed nerve. From previous discussion of the X-ray data it would seem that in spite of the freeze-sectioning and drying, or the dehydration with alcohol and subsequent embedding in polymer, the structural unit of myelin probably remains intact, and retains its radial orientation. The general appearance of the structure as seen in the electron micrographs would therefore be expected to be similar to that existing in fresh myelin.

In electron micrographs of sciatic nerve, the myelin shows a layered structure, the repeating unit apparently including one dense layer and one light layer. In the work of Fernández-Morán these layers gave a repeating unit of about 59 Å in the case of frog nerve and 71 Å for rat sciatic. These dimensions correspond very closely to one half of the fundamental repeating units calculated from the X-ray diffraction patterns of fixed and dried material. In the electron micrographs of fixed and embedded mouse sciatic nerve obtained by Sjöstrand a similar arrangement of broad, concentrically-wrapped bands was observed, and in addition a thin dark line was observed across the middle of the light layer. The repeating unit was, however, found to measure about 119 Å. No very extensive diffraction work has been carried out on mouse nerve or with embedded specimens, but rat sciatic nerve is found to give a repeating unit in the radial direction in the sheath which, under various conditions, is consistently 10 to 20 Å greater than that of the corresponding frog material. Furthermore, Dr. Sjöstrand very kindly made available for examination by X-ray diffraction the OsO_4-fixed and embedded specimen from which sections giving some of the best electron micrographs were obtained. This specimen gave two well-defined diffraction bands at about 74 Å and 148 Å, indicating a fundamental repeating unit in the radial direction in the sheath of about 148 Å. The repeating unit seen in these electron micrographs thus appears to be larger than one half of the unit cell deduced from diffraction data but yet appreciably smaller than the full unit cell dimension. The discrepancy may be due to the expansion or contraction of the structure during sectioning and examination in the electron microscope, or it may arise from the different techniques used for measuring. Even assuming that both methods give accurate measurements, there still remains the fact that the electron microscope selects certain portions of the structure for examination, and measurements are made only on the most clearly defined units, whilst in X-ray diffraction the whole sciatic bundle is used, and the dimension obtained should be representative of the greater part of the structure. One of the main difficulties encountered in using OsO_4 as a fixative is its slow and uneven penetration of thick specimens, and it is possible that the individual sheaths selected for measuring in the electron microscope may be the thinnest ones, which, other things being equal, would be the ones contributing least to the diffraction. Better agreement between E.M. and X-ray diffraction data may be obtained after refined fixation and embedding techniques have been applied in preparing the specimens.

The dense layers seen in electron micrographs of OsO_4-fixed nerve are probably produced by the deposition of osmium, and presumably indicate the locations of the groups which react with OsO_4. The definition and regularity of these layers is in agreement with the suggestion made from X-ray data of a precise ordering of the reactive groups in the radial direction. If two dense layers and two light layers are to be included in the fundamental repeating unit, then in spite of the enhancement of the first order diffraction, the deposition of osmium would appear to be at equivalent points in the two halves of the unit. However, the electron microscope does not give an accurate measurement of the density of deposition, nor is the distribution of osmium in the plane of the layers known. Perhaps the differences between alternate layers are to be found in the number and distribution of the reactive groups in the layers rather than in the general location of such groups in the radial direction. Also, in comparing E.M. and X-ray data, the limited resolution of the former must be considered. Sjöstrand has estimated the thickness of the main dense layer to be of the order of 30 Å but this is getting close to the limit of resolution of the present E.M. methods, and the possibility of this layer consisting of a number of thinner and possibly irregularly spaced layers or patches is not excluded.

Because of the present uncertain correlation of the E.M. and X-ray diffraction data and the lack of detailed knowledge of the groups reacting with OsO_4, a complete understanding of the fixation and its structural implications is not yet possible. A more closely co-ordinated electron microscope and X-ray diffraction study of normal and modified nerve myelin and related lipid structures is required.

SUMMARY

A study has been made of the effects of osmium tetroxide on the X-ray diffraction patterns of normal and modified sciatic nerve.

Intense and well-defined diffraction patterns were obtained from OsO_4-fixed fresh nerve but not from nerve which had been initially modified by temperature changes, or treatment with lipid solvents or formalin. The effect is thought to emphasize the precise arrangement of reactive groups in fresh myelin and the disorganization of such groups which accompanies modifications.

OsO_4-fixed fresh nerve appears to resist further modifications, and the structure revealed by the electron microscope probably resembles very closely the normal structure.

Detailed correlation of E.M. and X-ray diffraction data is not possible because of lack of knowledge of the precise chemical action of OsO_4, but some tentative suggestions are made.

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OBSERVATIONS ON THE SOCIAL BEHAVIOUR OF CELLS IN TISSUE CULTURE

II. "MONOLAYERING" OF FIBROBLASTS

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Many tissue culturists have noticed that in hanging drop cultures made with a liquid medium the migration of fibroblasts is restricted to the interface between cover slip and liquid. A sheet of cells which is mainly one cell thick grows out in this plane. It is generally agreed that the cells are confined to the interface because their mechanism of movement requires a solid substratum. There has not however, to our knowledge, been comment on the fact that a cell in a liquid medium culture is presented not only with the interface between the cover slip and the medium, but with another interface, between the exposed surfaces of neighbouring cells and the medium; and that this second interface seems little used by the migrating cells, at least in the early stages of a culture's life.

The absence of comment on this apparent restriction of cell movement is perhaps to be explained by an impression that the cells have little opportunity to move over each other's surfaces. They seem to be migrating at similar speeds and predominantly radially from the explant, and such a tendency to uniformity of movement must reduce the chances of two cells crossing one over the other. Measurements show, however, that the cells are not very highly correlated in their movements. We referred in our previous paper (1) to the wide variation in linear velocity between different cells at the same time in the same culture. Direction of movement also, as we shall show in a subsequent paper, is by no means uniform, although the predominant direction is away from the explant. We therefore suggested in our previous paper that in liquid medium there is some restriction on freedom of movement of fibroblasts over each other's surfaces, which is partly or wholly responsible for the tendency to form a single layer of cells at the interface, a mode of behaviour which we termed "monolayering". The purpose of the present paper is to investigate monolayering further, and to bring forward evidence that it is in fact due to a mutual restriction of movement, with perhaps far-reaching implications for the analysis of cell behaviour.

Such tendency as there is for neighbouring fibroblasts to move in the same direction at the same speed may itself, like the monolayering, be an outcome of the suggested mutual restriction of movement. At the outset it is however necessary to take account of the possibility that such correlated movement is an outcome of other influences. These other influences, if they exist, must then contribute to the occurrence of monolayering, and this would greatly complicate the investigation. The difficulty can be largely avoided if we ensure that all the fibroblasts observed do not move predominantly in the same direction by placing two explants close together in a culture. By this means we make two advancing sheets of cells move towards each other and collide in the space between the explants. This is the experimental situation we have used throughout the present work.

METHOD

Cultures were made by the hanging drop technique in equal amounts of (a) fowl plasma and (b) extract of 7-day chick embryos diluted to 25 per cent with Pannett and Compton's (7) Saline. Explants were fragments of the ventricle of 7 to 10 day old chick embryos. Two explants were placed 0.5 to 1 mm apart on each cover slip. The plasma clot between the explants was cut away before incubation. The space so caused filled immediately with exudate, and this inter-explant region was the site of most of our observations. Those on living cultures were made by phase contrast microscopy at 38°, in some instances with the help of time-lapse filming; those on killed cultures after fixation in 4 per cent formaldehyde-saline and staining in alum haematoxylin.

RESULTS

The following is a brief qualitative description, based mainly on the examination of films, of what happens between the two confronted explants in what we shall term the inter-explant area. The two sheets of cells, each a constantly changing irregular two-dimensional meshwork, looser in texture at the periphery than centrally, advance towards each other, narrowing down the empty space between. Isolated cells may cross over from one sheet to the other. Contact is then established here and there between the peripheral cells of the two sheets and steadily becomes more general. The loose texture of each periphery is gradually lost, the intercellular spaces becoming smaller. So far there is no noticeable diminution of the steady trend of the two sheets outward from their respective explants. But as empty spaces fill, concerted movement becomes more localised, except at the lateral margins of the inter-explant area, i.e. the sides not bordered by the explants. There some of the cells take part in a new trend towards the lateral unoccupied space. As the intercellular spaces become reduced to sizes considerably smaller than the area of a single cell, general trends of movement cease to be detectable, except laterally. The cells maintain only an uncoordinated oscillation. The continuous sheet of cells which now covers the inter-explant area is still largely a "monolayer", though with some overlapping of cell processes and here and there of entire cells.

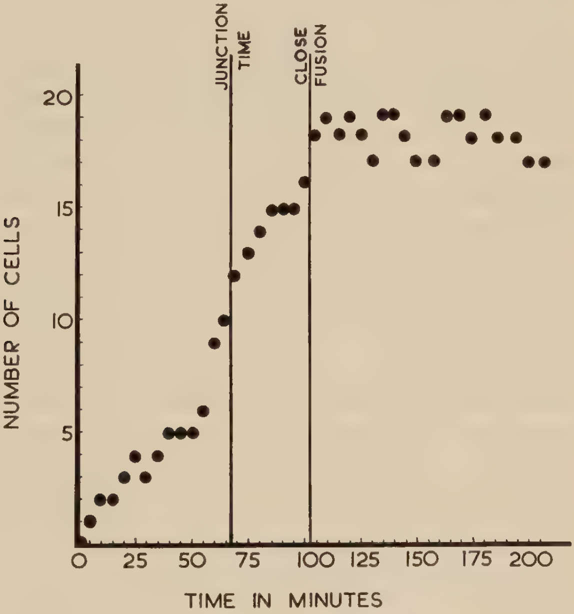

Fig. 1. The number of cells in an area of interface between two explants during the approach and junction of the two sheets of cells, recorded at five-minute intervals. "Junction time" is the time when opposing cells first make contact; "close fusion" indicates the time when the cells have become closely packed throughout the area observed.

For the validation and further analysis of these qualitative impressions we have undertaken several different measurements of cell behaviour in the inter-explant area.

Cell population. The number of cells observed within a square area of the interface, 0.12 mm^2 in extent, situated in the centre of the inter-explant area, has been counted at five-minute intervals during the approach and junction of the two sheets of cells in a number of cultures. A typical example is shown in Fig. 1. In this culture readings were started just before any cells had entered the area of observation. As cells invaded from both sides, the population of the area rapidly and steadily rose. The time at which the first junction between cells of the two sides was established in the observed area is shown by the first vertical line ("junction time"). The population continued to rise for a period after the junction time, the cells becoming more tightly packed, but when no space bigger than a cell remained ("close fusion") it appeared to stabilise.

Eight culture areas have been followed in this way from the time of the first appearance of cells in them to long after the time of close fusion. The regression coefficients for population against time have been calculated for each culture before and after first junction. Of the readings before junction, which start as soon as cells enter the observed area, the first quarter have been discarded, because one or a few scattered peripheral cells frequently enter the area in advance of the main influx, so that the initial increase of population may be very slow. Since increase of population continues undiminished for a period after the junction time, while the larger free spaces are filling, the first hour after the junction time has been arbitrarily rejected from the calculations, and the post-junction regression based on the subsequent readings. The post-junction regression coefficient has been subtracted from the pre-junction regression coefficient for each culture. The mean of these differences was 0.21 ± 0.07 (the pre-junction coefficients being larger) which is significantly different from zero ($t = 2.88$, 7 degrees of freedom, $0.02 < P < 0.05$). Our sample of cultures therefore clearly shows a check in the rise of population in the inter-explant area after junction of the two sheets of cells has occurred (Table I).

The mean regression coefficient before junction (0.32 ± 0.07) is significantly different from zero ($t = 4.9$, 7 degrees of freedom, $P < 0.01$). After junction it is 0.11 ± 0.05 which is not significantly different from zero ($t = 2.3$, 7 degrees of freedom, $0.05 < P < 0.1$). Nevertheless we consider that more extensive data would show a significant increase in population of the post-junction group. This is partly because the necessarily arbitrary definition of the group means that some cultures will be included which are still filling up vacant spaces, partly because we have other evidence that a very slow immigration may continue even when packing has become very close, and partly because mitosis continues. The population therefore probably does not stabilise completely after junction, but it very nearly does.

A check in population increase would of course eventually occur if two monolayers of cells simply continued migration regardless of each other after they had met. In that case the population would stabilise at a minimum of approximately twice the level it had reached at first junction. Twelve cultures were available in which the level of apparent stabilisation (i.e. the mean level in each culture where at least the last 4 available readings showed no upward trend) could be compared with the population level at the time of first junction (Table I). Putting the latter at 100, the mean level of apparent stabilisation was 158 ± 7, which is significantly different from 200 ($t = 5.9$, 11 degrees of freedom, $P < 0.001$). The check in population increase appears therefore to be inconsistent with the superposition of two monolayers; nor was any suggestion of this detectable by watching the films.

TABLE I

Rate of increase with time of cell population in an area between the explants, before junction of the outgrowing cells, and some time after junction. For the same cultures, and for four additional ones, the population within the area at the time of junction (mean of the readings between which junction occurred) and when approximate stabilisation has occurred (mean of several readings) are given in the last two columns.

Regression of population on time			Population	
Before junction	After junction	Difference	At junction	At stabilisation
0.373	0.186	0.187	12.0	17.8
0.530	−0.083	0.613	16.5	18.9
0.259	0.057	0.202	11.0	16.7
0.249	0.333	−0.084	10.5	18.0
0.349	0.078	0.271	11.0	16.8
0.411	0.018	0.393	11.0	17.5
0.201	0.048	0.153	11.0	15.5
2.208	0.230	−0.022	7.5	14.0
–	–	–	8.5	18.1
–	–	–	10.5	14.7
–	–	–	9.5	13.5
–	–	–	11.0	17.9

Observation of the films makes it clear that the main check to population increase occurs after junction. It is important to consider whether any foreshadowing of the check can be detected before the first junction is made. Thirty-three cultures were followed up to their junction time for this purpose. For each culture the regression of population on time was calculated in the pre-junction period, discarding as before the first quarter of the readings, which eliminates any initially slow rise, and also omitting from the calculation the last quarter of the readings just before junction when mutual interference might be expected to make itself felt. By extrapolation from this regression the expected population at the observed junction time was calculated and compared with the actual population at this time. The mean difference (expected minus observed population) was 0.07 ± 0.60, which is obviously not significant. We were therefore unable to detect any check to population growth before junction had occurred: the population at first junction can be effectively predicted from the slope of the steady population rise some time before. The mean observed population was 12.0 so that the 5 per cent confidence limit of the difference is about 10 per cent of the mean.

The observed check in the steady rise of population in an area between the two explants must occur either by cells starting to leave the observed area nearly as fast as they enter it, or by cells almost ceasing to enter it. One obvious possibility may be disposed of at once: that cells start to leave the area by dropping off the cover slip, and are replaced by continued migration from the explant. In fact, not one of the many hundred fibroblasts which have been followed for long periods after junction has ever been lost in this way; nor are cells found loose in the medium after culture.

Velocity. The velocity of cell movement was measured directly in living cultures by plotting the position of a given cell every five minutes from an eye-piece grid on to graph paper; a magnification in the microscope of 120 times was used, and the distance between every pair of successive points measured. Readings were made in 10 cultures some time after junction of the two sheets of cells, when the population in the inter-explant area had become closely packed. Each culture provided velocities of several cells, in two positions; (a) between the explants and (b) in the freely expanding sheet of cells, also in an area of liquid medium, at either side of the pair of explants. Cells in both situations were used only if they were in contact with 4 or 5 other cells, in order to standardise the known effects of contact on velocity (1). In situation (a), mean velocity (305 measurements) was 11.1 ± 0.7 μ/hour; in situation (b) (241 measurements) it was 41.9 ± 1.8 μ/hour. The difference is obviously significant. The mean speed in situation (b) compares satisfactorily with that obtained by a different method in our previous work (1) for cells in a freely moving sheet in the inter-explant area before junction; mean velocity for a contact number of 4, 4.5 or 5 was there 49.3 μ/hour.

The velocities in the inter-explant area after junction are so low that the question must arise as to whether the cells are in fact stationary, the recorded movement consisting merely of errors of observation and measurement. This is not so, since in films of cultures in which the two sheets of cells had joined closely together the cells can be seen to be moving independently, and very occasionally to change their immediate neighbours. The variations in the cell population within a given area which occur after close packing has been attained are also evidence of continued movement.

Direction of movement. The frequency with which a cell moves in a given direction has been estimated by plotting the course of the cell as for the velocity measurements (but a magnification of 320 times was used) and recording the direction of the line connecting its position at the beginning and end of each five-minute interval. The direction was noted as falling within one of four quadrants: away from the explant, towards it, to left or to right. The same cells (15 cells from 8 cultures) have been observed both before and after junction, so that it is known from which explant they have come. Of 310 observations made up to the time of junction (behaviour is unaltered right up to the time of junction), movement was away from the explant in 145, to the left in 87, to the right in 64 and towards the explant in 14. These frequencies differ of course significantly amongst themselves ($\chi^2 = 114$, 3 degrees of freedom, $P < 0.001$). Of 126 observations made more than one hour after junction of the two sheets of cells had first occurred, movement was away from the explant in 36, to the left in 33, to the right in 35 and towards the explant in 22. The distribution of movements after junction is significantly different from that obtained before ($\chi^2 = 28$, 3 degrees of freedom, $P < 0.01$). Furthermore, after junction the frequency of movement does not differ significantly as between the four directions ($\chi^2 = 4.0$, 3 degrees of freedom, $P > 0.2$). We believe, however, that more extensive data and more refined analysis would show a significant though slight excess of movement away from the explant at the expense of movement towards it, for the same reasons that led us to consider that complete stability of population after junction would not be demonstrable.

It might be argued that the observed decrease of velocity would itself produce, as an artefact, the equalisation of directional frequency, since random errors of measuring technique become more important the slower the speed. The plotted tracks of the cells give no indication, however, that after junction there is any trend away from the explant underlying the observed irregularity of direction. It may be added that after junction the cells do not circle or undergo any other detectable patterned movement which could produce the equal distribution of directions actually observed.

We may therefore conclude that after junction movement ceases to be mainly away from the explant, and comes to differ little more than randomly from equality in all directions. In combination with the slowing of velocity which occurs after junction, we have here the probable explanation of the check in population increase in the inter-explant area: cells substantially cease to enter or to leave the area.

We now turn to consider the causes of the change of behaviour at the time of junction. It is necessary first of all to dispose of the possibility that the change merely reflects the cessation of "growth" which marks the end of the life-span of a culture. (Our data on cell velocity have already provided some evidence against this.) Measurements of the radial extent of the sheet of cells which had migrated from each explant were taken shortly before junction in 6 cultures, when on the average four fifths of the inter-explant area had been covered by the migrating cells. Measurements taken at the open sides of the two explants showed no significant difference from those taken in the inter-explant area, averaging 310 μ and 324 μ respectively; the mean difference between these measurements on the 12 explants was 14 ± 27 μ. Measurements made on 8 other cultures earlier before fusion showed the same equality; growth appears to be substantially the same on all sides of an explant up to the time of junction. Three to 18 hours after junction, the radial extent at the sides of the explants averaged 640 μ and much exceeded half the distance between the two explants which averaged 407 μ; the mean difference of 12 pairs of measurements was 234 ± 95 μ. Lateral outgrowth after junction had therefore significantly surpassed inter-explant outgrowth in extent ($t = 2.5$, 11 degrees of freedom, $0.02 < P < 0.05$). But since the packing of the cells was no longer the same in the two situations, a more stringent test is given by comparing, in cultures 3 to 18 hours after junction, the number of cells in a strip of given width between the two explants with the number in a strip of similar width extending to the periphery of the outgrowth at the sides of each explant. Two such estimates were usually made at the side of each explant; the position of the strips was selected "blindly" without reference to the extent of growth. Data from 18 explants are available and only one showed fewer cells at the sides than in the inter-explant area. In the lateral strips the population averaged 1.5 times that of half the inter-explant strip, and the difference is significant ($t = 4.5$, 17 degrees of freedom, $P < 0.001$). The change of behaviour between the two explants which brings migration into this area almost to a standstill shortly after junction is not therefore merely a reflection of the ageing of the whole culture; migration clearly continues in the rest of the culture.

Can the behavioural change in question be due to a particular pattern of metabolite concentration established between the two explants? An effect of the absolute concentration of some stimulatory or inhibitory substance which the explants consume or produce is the first possibility. It must be rejected, because half-way between the two explants at the time of junction the concentration of a metabolic product cannot significantly exceed, nor the concentration of a consumable metabolite fall below, the concentration in the immediate neighbourhood of the explant. Yet concerted movement away from an explant is not inhibited in its immediate neighbourhood, either in the inter-explant area just before close fusion or at the sides after close fusion. A second possibility is that we have to deal with absolute metabolite concentrations engendered exclusively by the outgrowing cells. But these should operate as much in the densely populated part of a migrating sheet of cells as in the dense population after junction; yet one moves with an active trend and the other does not. Absolute concentrations can therefore hardly be invoked in any form. There remain concentration gradients. If the two explants either consume or produce some substance, the concentration gradient of the substance will be zero along a line between the two explants parallel to their confronted faces. An all-or-nothing directional response to such a gradient would pile the cells up at the line of zero gradient; this does not occur. We can also exclude the more likely kind of reaction to such a gradient, *viz.*, that the intensity of the directional response of the cell varies with the slope of the gradient. It this were so, the diminishing slope should slow the advance of the cells before they meet, so that junction should be delayed or incomplete for a time. This is of course precisely how monocytes behave in such a situation (2), though apparently the implications of this behaviour have not previously been pointed out. Fibroblasts behave quite differently. Their change in behaviour begins only after contact has been made, as shown for increase of cell population in an area, radial extent of outwandering, frequency of the different directions of movement and (1) velocity of displacement of individual cells.

Since the influence of diffusible substances in the medium seems incapable of providing a plausible explanation of our observations, and the same arguments would apply to any action at a distance, it seems that the actual junction of the two sheets of cells must be responsible for the change in behaviour which follows. There seems no other likely explanation than our original supposition that there is a restriction of the freedom of movement of cells over each other's surfaces. When they are faced with a situation such that in order to continue their previous trend they must move over each other, they cease their previous trend and remain as a monolayer. In order to support this hypothesis, we must now produce evidence that after junction of the two sheets of cells a tendency to form a monolayer really occurs. That is certainly the impression derived from qualitative observation, but some overlapping of cells takes place. This makes it necessary to compare quantitatively the actual distribution of cells with the distribution which might reasonably be expected if there is no restriction on overlapping of cells.

Since after junction the cells continue moving, though slowly; and since they are not moving in an orderly way, the directions in which they are going at any moment being uncorrelated; it may be supposed that if they did not interfere with each other's movement they would be, or would soon become, randomly distributed in two dimensions, with a certain number of the cells overlapping when viewed in plan. If they are assumed to be randomly distributed in this way, the expected number of cell overlaps can be calculated for a given cell population in a given area. By a cell overlap we mean any instance where one cell is partly or wholly superimposed on another cell. For convenience we have used overlapping of nuclei rather than of whole cells. The nuclear area affects the chances of overlap occurring, and for the calculation we require therefore to know the *effective area* of a nucleus, that is, the area such that if the mid-point of another nucleus falls within it an overlap will occur. In each culture, fixed some time after junction had occurred and stained, the mean diameters of a large sample of nuclei were measured in an expanse of known area between the two explants. The mean nuclear radius, r, being used, the effective area of a nucleus is given by $\pi\ (2r)^2$ (assuming that nuclei are circular in plan, and disregarding the variance of their radii). The chances of overlap of any pair of nuclei in a given expanse of culture are then given by the effective area of one nucleus divided by the total area (A) of the expanse of culture observed. If the given expanse contains n cells (the number of cells is easily counted), the number of possible pairs of cells is $\frac{n\,(n-1)}{2}$. The expected number of overlaps for a given area of any culture can therefore be calculated from the expression $\frac{\pi(2\,r)^2}{A} \cdot \frac{n\,(n-1)}{2}$ and compared with the actual number of overlaps observed. Observed and expected values were obtained for 24 areas in 9 separate cultures, fixed from 3 to 24 hours after first junction (Table II). The observed values average 0.33 of the expected.

We are indebted to Dr. N. W. Please for pointing out how this difference should be tested for significance. Since the expected value may be assumed to be Poisson distributed, its variance is equal to itself, and the difference may be tested by $\frac{\text{exp.} - \text{obs.}}{\sqrt{\text{exp.}}}$, the probability of which may be read in a

TABLE II

Overlapping of nuclei in the inter-explant area after junction of the outgrowths. The first column shows the total number of cells in the area observed. The effective area of one nucleus is given as a percentage of the total area observed. For calculation of expected number of overlaps, see text. The final column is the difference between observed and expected number of overlaps divided by the standard deviation of the expected number. In no case does the probability that the difference occurs by chance exceed 0.01.

No. of cells	Effective nuclear area	Expected no. of overlaps (E)	Observed no. of overlaps (O)	$\frac{E-O}{\sqrt{E}}$
148	0.64	69.2	20	5.91
177	0.57	88.4	22	7.06
152	0.51	58.1	10	6.31
163	0.54	71.8	24	5.64
131	0.82	70.0	17	6.34
133	0.57	50.4	12	5.41
123	0.60	44.9	9	5.36
183	0.71	118.1	35	7.65
149	0.53	58.2	22	4.75
138	0.54	51.0	16	4.90
85	0.59	21.0	6	3.27
122	0.72	53.1	20	4.54
126	0.54	42.4	8	5.28
200	0.46	90.9	56	3.66
232	0.51	136.3	70	5.68
260	0.47	158.6	75	6.64
147	0.72	77.7	25	5.98
168	0.65	91.3	26	6.83
126	0.63	49.3	12	5.31
244	0.55	161.8	50	8.79
286	0.49	200.2	85	8.14
110	0.94	56.2	32	3.23
154	0.53	62.0	38	3.05
130	1.10	92.9	39	5.59

table of normal deviates. Every observed value proves to be significantly less than its expected value at the 0.01 level of probability. Indeed, if the expected value is made half that calculated above, the population of observed values is still significantly less, i.e. the mean of the normal deviates used for testing differs significantly from zero ($P<0.001$). The distribution of cells in these cultures is then such that many fewer nuclear overlaps occur than is to be expected from a random distribution. The evidence therefore supports the hypothesis of a restriction of free movement of fibroblasts over each other's surfaces.

DISCUSSION

Our results lead to the conclusion that there is some restriction of the movement of fibroblasts over each other's surfaces. It is a restriction which does not apparently operate at a distance to hinder the mutual *approach* of fibroblasts. Fibroblasts therefore freely make contact with each other, and in fact adhere together (5), forming the meshwork so characteristic of their growth. The restriction operates only after contact has been established. There is evidence (1) that the initial reaction to contact is a slight acceleration of the movement of the cells towards each other. After that there must usually occur a prohibition of further movement in this direction. The prohibition does not invariably occur in the conditions we have investigated, since there develops some overlapping of cells; and we have as yet no conclusive information as to how important the prohibition is in cultures grown for more than 36 hours, or grown in different media.

This directional prohibition of movement we shall refer to briefly as "contact-inhibition". Two hypotheses may be suggested as to how it works: (a) The surface activity by which a fibroblast moves may be inhibited in the neighbourhood of the place on its surface where an adhesion has formed with another fibroblast. (b) The exposed surface of a fibroblast parallel to the cover slip may be of such a kind that no adhesion or spreading of another cell surface can usually occur on it; thereby it would differ from the lateral surface by which the cells adhere to each other. A differentiation of some kind between exposed and mutually adherent cell surfaces, with which this phenomenon might be compared, occurs normally in epithelia. At present no decisive evidence can be offered for either of these hypotheses.

By discouraging movement in certain directions contact-inhibition may, as we previously suggested (1), be an explanation of the inverse relationship which we found between velocity and number of contacting cells. We suggested another explanation of this inverse relationship in the mutual mechanical interference caused by adhesion amongst discordantly moving cells. This is a mode of cell interaction which should be kept distinct from contact-inhibition. Distinct too are the repulsive effects discovered by Twitty (8) and his colleagues in neural crest cells. These latter effects are mediated by diffusing substances, and therefore act at a distance. We believe that a similar sort of mutual restriction of movement by diffusing substances may also be important in the behaviour of macrophages; but we have not been able to demonstrate it in the behaviour of fibroblasts.

We must now outline some of the consequences which contact-inhibition may have for the explanation of fibroblast behaviour in liquid media. In the first place, contact-inhibition must make cells move, if they move at all, predominantly away from the explant. Movement which involves overlapping will be discouraged, movement into spaces clear of cells will be preferentially selected, and hence there will be a general trend of migration into the open spaces around the explant. The commonly accepted postulation of diffusion gradients is not then required to explain why cells spend most of their time travelling in one main direction in relation to the explant—away from it. The direction of movement is controlled not by the position of the explant but by the position of free space.

A second inference now follows: whatever the original shape of a culture, it will, as it grows, tend to assume a circular form (when seen in plan). This must happen because cells tend to move towards free space; the direction of movement in which they interfere least with each other is at right angles to the culture periphery. If there is an indentation in the periphery of a culture, cells will converge into it from both sides, and proceed to fill it. If there is a projection from the periphery, cells will disperse from it to both sides, and reduce its prominence. In the first case the number of cells lying within the segment of the culture defined by the indentation will increase more rapidly than the number in neighbouring segments. Conversely, in the second case it will increase less rapidly. Only a circular form will maintain itself, because only then is each part of the periphery of the culture situated on the same radius as the free space it will invade. While contact inhibition can in this way account for much of the tendency to a circular form, further observation will probably show that the process has additional complications.

The long-standing puzzle as to why fibroblast cultures rapidly assume and regenerate to a circular form has been approached previously by experiments on cultures in fibrin clots (3, 4, 6). Without further evidence obtained in such different conditions we will not discuss this work, but we believe that here too the hypothesis of contact-inhibition may be of explanatory value. We also require additional information as to the conditions in which contact-inhibition can occur before we apply it to problems of the distribution of cells in the whole organism, such as the lamellar arrangement so often found in connective tissue, and to the starting, directing and stopping of migratory movements in embryo and adult. Some of the latter problems have been discussed by Weiss (9). Our "contact-inhibition" clearly comes within the general category of Weiss' "coaptation".

SUMMARY

1. Observations have been made on cell behaviour in the area between two embryonic chick heart explants, placed 0.5 to 1 mm apart in liquid medium tissue culture.

2. The cell population in a given area in this situation rises steadily from zero as cells from both explants invade it, but then almost ceases to rise soon after the opposing outgrowths have met. There is no detectable diminution of rate of rise of population while the cells are approaching, before the outgrowths actually join.

3. The velocity of individual cell movement after junction of the outgrowths is much slower between the explants than at either side of the explants.

4. The direction of movements of cells in the region between the explants is predominantly outward from their respective explants until the two outgrowths of cells join, when it becomes little more than randomly different from equal in all directions.

5. It is concluded that outgrowth into the area between the two explants almost ceases soon after junction has been established between the opposing sheets of cells. After this junction, the number of cells in a radial strip joining the two explants becomes exceeded by the sum of two radial strips one at the side of each explant. Culture outgrowth therefore continues laterally after it has substantially ceased between the two explants.

6. After junction the cells between the two explants tend to remain as a "monolayer". The number of instances where one nucleus overlaps another is much fewer than is to be expected if the cells were randomly distributed.

7. It is concluded that fibroblasts avoid moving over each other's surfaces. Such "contact-inhibition" of movement can explain why it is that fibroblasts normally migrate predominantly radially from an explant and that the whole culture tends rapidly to become circular in plan whatever its initial form.

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THE EFFECT OF LYSOZYME ON THE ELECTROPHORETIC MOBILITY OF CERTAIN COCCI[1]

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Lysozyme is believed to cause the hydrolysis of a specific muco amino polysaccharide present in bacteria susceptible to lysis by this enzyme. This polysaccharide is believed to be located in the cell wall and to play an important part in the permeability of this structure (1, 5). Since the external surface of the cell possesses an electrical charge which may be important in staining, adsorption, and other phenomena associated with the colloidal properties of the cell, it is of interest to know whether the cellular substrate of lysozyme contributes to the electrical charge of the cell. This investigation was undertaken to compare the electrophoretic properties of normal cocci with cells which had been subjected to lysozyme treatment.

EXPERIMENTAL PROCEDURE

As complete lysis of the bacterial cells renders them invisible under the electrophoretic microscope, it was necessary to screen available cultures to find strains in which the specific lysozyme substrate of the cells would undergo hydrolysis to the extent of releasing reducing compounds in the presence of the enzyme but without the cells immediately undergoing lysis. After study of some twenty strains of bacilli and thirteen strains of cocci, three organisms were selected from the stock collection of the Department of Bacteriology, Kansas State College, as being best suited for study. These were *Micrococcus lysodeikticus* A.T.T.C. 4698; *Micrococcus albus*, isolated from milk; and *Sarcina lutea*, isolated from the air of the laboratory. Cells for these studies were grown on the agar medium suggested by Smolelis and Hartsell (6). After twenty-four hours' growth at 32°C cells were harvested, washed three times in 0.01 M potassium phosphate by centrifugation, and suspended in the same medium buffer at the desired pH. Crystalline egg white lysozyme (Armour) was dissolved in 0.01 M KH_2PO_4. For experiments herein reported the final concentration of the added lysozyme was 10 micrograms per milliliter of the buffer-cell suspension.

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[2] Portion of a dissertation presented by the senior author as partial fulfillment of the requirements of the degree Doctor of Philosophy in Bacteriology at Kansas State College.

Electrophoretic measurements were made following the usual procedure (3). The buffer solutions were kept at a constant ionic strength (0.01M) and all measurements were made at 25°C.

DISCUSSION OF RESULTS

Since Harris (3) has shown that the pH of the suspending medium is important in detecting small changes in the electrical charge of bacterial cells, comparisons of electrophoretic mobilities of normal and lysozyme-treated cells were made using buffers having pH values of 3.4, 4.5, 5.0, 6.0, and 6.5. Results of a typical experiment are shown in Table I. Statistical evaluation of the data by the t-test of Fisher (2) shows a significant (1 per cent level) reduction in the mobility of cells exposed to lysozyme for one hour for all three organisms at all pH levels except pH 3.4 for *Micrococcus lysodeikticus*. Although the differences were significant it is apparent that the change in the over-all charge of the cell was altered only slightly by the lysozyme. This would indicate that the polysaccharide substrate attacked by the lysozyme may contribute only slightly to the zeta potential of the organism. Egg white lysozyme possesses a positive charge (4) and would be expected to cause some reduction in the over-all negative charge of the bacterial cell when it combines with it. In order to demonstrate this effect, lysozyme was inactivated by heating at 100°C for 30 minutes and then added to the cell suspensions. The electrophoretic behavior of normal cells, cells plus inactivated lysozyme, and cells plus inactivated and active lysozyme are indicated in Table II. Apparently, the inactivated enzyme was still adequately adsorbed by the bacterial cells to cause significant reduction in the mobility of the cells. However, further reduction in the mobility of the cells occurred when active lysozyme was added to the suspension.

A time-rate series of experiments was carried out to determine whether the reduction in the electrophoretic mobility indicated in Table I and Table II was due to the presence of the lysozyme *per se* (as indicated by decreased mobility with heat-inactivated enzyme) or to some change in the cellular surface produced by the lysozyme. The results obtained with *Sarcina lutea* at pH 5.5 are shown in Fig. 1. The normal cells and those treated with the heated enzyme showed constant mobility rates over the 90-minute experimental period. In contrast, the cells in the presence of the active lysozyme showed a rapid decrease in movement during the first twenty minutes of the experiment. This sharp decline in mobility was followed with an apparent increase and then a gradual decreasing rate for the duration of the experiment. This type of curve with a more or less pronounced peak during cer-

TABLE I

Mean mobility values of normal and lysozyme-treated cells expressed as microns/second/volt/centimeter when measured at different pH levels.

Organism	Treatment	Mobility as μ/sec/v/cm at pH				
		6.5	6.0	5.0	4.5	3.4
Micrococcus lysodeikticus	none	3.51	3.55	3.38	3.34	2.59*
	+lysozyme	3.27	3.20	3.23	2.90	2.59*
Micrococcus albus	none	4.71	4.67	4.79	4.57	4.07
	+lysozyme	4.15	4.21	4.10	4.01	3.75
Sarcina lutea	none	3.31	3.14	2.65	2.60	1.97

* Values not showing significant (1 per cent level) differences due to treatment at 39 degrees of freedom.

TABLE II

Results of experiments showing the effect of heated and unheated lysozyme on the electrophoretic mobility of three bacterial species.

Organism	Mobility of the cells as microns/second/volt/centimeter at pH 5.4 when treated with			
	buffer only	heated lysozyme	unheated lysozyme	heated plus unheated lysozyme
M. lysodeikticus	4.5	3.9	4.2	3.2
M. albus	4.8	4.4	4.3	3.6
S. lutea	4.5	4.1	3.7	3.6

tain times of measurement was observed consistently and with each of the three strains of cocci studied. It may indicate some type of reversible change occurring in the cell surface as a result of the lysozyme activity.

The results indicate that the changes in electrophoretic mobility of cells exposed to lysozyme were due to slight changes in the cell surface caused by the enzyme activity. It should be emphasized that in all the experiments reported in this investigation, the reaction of the suspending medium was kept acid and the concentration of lysozyme was kept low so that the cells did not undergo lysis to the extent that they could not be seen distinctly in an unstained condition under the microscope. Changes in charge of a greater magnitude might be expected during actual lysis of the cells.

The fact that the electrical charge of the cells appears to be altered following

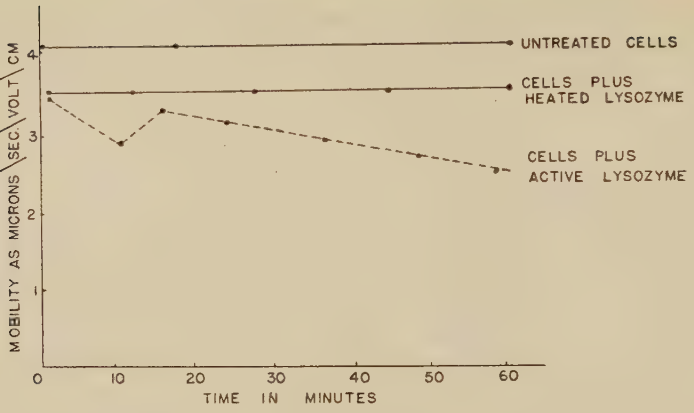

Fig. 1. Time-rate measurements of the electrophoresis of *Sarcina lutea* treated with heat-inactivated lysozyme and active lysozyme, pH 5.5.

treatment with lysozyme gives additional evidence as to the location of this polysaccharide material in the bacterial cell. Since this material seems to be important in the structural make-up of the cell wall (1) it might be expected that it would be located in the outer portions of the bacterial cell. Further support is given this idea by the suggestion of Peniston and Harris (5) that treatment of resting cells with lysozyme increases the permeability of the cell to glucose during respiration. Studies are in progress relative to the part this lysozyme substrate may play in staining and adsorption phenomena.

SUMMARY

Small but significant decreases in electrophoretic mobility of *Sarcina lutea*, *Micrococcus lysodeikticus* and *Micrococcus albus* occurred when the cells were subjected to the action of lysozyme. Heat-inactivated enzyme apparently combines with the cells causing a different type of change in the electrophoretic behavior of the cells. These findings are discussed in relation to the location of the substrate of lysozyme in the structure of the cell.

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THE EFFECT OF COBALT ON MITOSIS IN TISSUE CULTURE

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During investigations into the distribution of zinc and cobalt in normal and neoplastic animal tissues, it was found that radioactive Zn^{65} and Co^{60}, injected into mice subcutaneously, were taken up by all tissues examined, and were also present in significant amounts in the deoxyribosenucleoproteins from tumours in these animals (Zn[2], Co[3]). It had previously been shown (1) that deoxyribosenucleoproteins from similar tumours in untreated animals and from other normal animal tissues contained a measurable amount of natural zinc. It therefore seemed interesting to determine whether a small excess of either of these metals would in any way affect the behaviour of intracellular nucleoproteins and hence produce specific cytological effects when added to the medium in which vertebrate tissues were cultivated. In this paper the effect of cobalt (as chloride) on cultures of embryonic chick tissue is described. It will be shown that, somewhat unexpectedly, the metal affects mainly the nucleolar ribosenucleoprotein system and has no observable effect on the ribose and deoxyribosenucleoproteins in other parts of the cell.

Two series of experiments were made. In Series 1 the tissue was transplanted into a medium containing cobalt in various concentrations; in Series 2, the explant was allowed to grow in normal medium for 24 hours, at which time mitosis was approaching its maximum, and a solution of cobalt in a mixture of serum and Tyrode's solution was then deposited on the growing tissue.

MATERIALS AND METHODS

Cultures of the heart from 11–12 day chick embryos were used for all experiments except for some in Series 1 for which frontal bone of the same age was cultivated. Two types of cultures were made:

1. Ordinary hanging drop cultures. In these the tissue fragment was explanted directly into a clot composed of equal volumes of fowl plasma and chick embryo extract. The volume of the clot was standardised at 0.025 ml. The chick embryo extract was made by mixing 1 volume of chick embryo pulp with 2 volumes of Tyrode's solution and taking the supernatant after centrifuging. When this type of culture was used the tissue was usually subcultivated once before treatment with cobalt.

2. Horseshoe cultures. In these, the piece of chick heart was explanted into a fluid

medium lying within a horseshoe-shaped clot. The fluid was obtained by incubating a mixture of equal volumes of fowl plasma and chick embryo extract for 10–20 minutes, breaking up the clot so formed and re-incubating for 1 hour; the exudate or fluid phase was then removed with a pipette. The horseshoe-shaped clot was made from a mixture of equal volumes of fowl plasma and chick embryo extract. The volume of the clot was standardised at 0.012 ml and the fluid phase at 0.003 ml. This method was devised by Dr. Honor B. Fell to give free access to the cells in micro-injection studies on which the author was engaged, and for the present experiments had two important advantages:

(a) The cells in the outgrowth were much flattened and formed a thin sheet which was excellent for detailed observation by phase contrast and made very fine preparations when fixed and stained.

(b) The added cobalt had less chance of being bound in an inactive form by the proteins of the clot.

In the experiments of Series 1, in which the tissue fragment was explanted into a medium containing added cobalt, the metal was introduced as follows. For hanging drop cultures a small known volume (< 0.3 ml) of an aqueous solution of cobalt chloride of known strength was run to the bottom of a small pyrex test-tube, evaporated to dryness and sterilised by dry heat at 140°C for 1 hour. 2 ml of plasma was then added to dissolve the cobalt chloride deposited in the tube and equal parts of this cobalt-containing plasma and ordinary chick embryo extract were used to form the standard clot. For horseshoe cultures a known amount of cobalt chloride from less than 0.15 ml of an aqueous solution was deposited at the bottom of a pyrex tube and sterilised as above. 0.1 ml of clot exudate was then added to dissolve the cobalt chloride and 0.003 ml of this cobalt-containing exudate was used to form the standard fluid phase in each horseshoe culture. For all experiments in this series, adequate control cultures were made with fowl plasma, embryo extract, and, for the horseshoe cultures, clot exudate identical with those used in the cobalt-treated cultures but without the addition of cobalt. In an experiment at any particular concentration of cobalt 6–12 cultures were prepared in medium containing added cobalt and the same number in medium without added cobalt. Cultures in this series were allowed to grow for 48 hours and then fixed and stained.

In the experiments of Series 2, 12–24 cultures of either type were grown in untreated medium for 24 hours. At the end of that time not less than 12 cultures were opened and in half of these 0.01 ml of a mixture of equal volumes of Tyrode's solution and fowl serum was deposited on the explant from a pipette; in the other half, 0.01 ml of an identical mixture of Tyrode's solution and fowl serum but containing additional cobalt as cobalt chloride (prepared as in Series 1) was placed on the explant. The opened cultures were resealed with hot paraffin wax and returned to the incubator. In some of these experiments a few cultures were passed right through the experiment unopened and untreated in any way. The cultures were fixed at various intervals up to 24 hours after treatment with the Tyrode-serum. In some of the experiments of Series 2, the cultures were mounted on special metal rings (4) instead of on the customary hollow ground slides, to permit of phase contrast observation of the living culture in the warm chamber; they were treated with Tyrode-serum and Tyrode-serum containing added cobalt as described above.

Cultures were fixed either in Maximow's fluid, in absolute methyl alcohol or in 5 per cent acetic alcohol. For each culture particular care was taken to transfer it rapidly direct from the incubator to the fixative to avoid changing the mitotic picture. Each culture was vigorously agitated in the fixative for 1 minute and then transferred to a fresh bath of the solution. By these means rapid and good fixation was achieved. Cultures fixed in Maximow's fluid were stained in Ehrlich's haematoxylin; those fixed in methyl alcohol were stained either with May-Grünwald-Giemsa by the method of Jacobson and Webb (6) or with methyl-green pyronin (0.6 per cent methyl green B.D.H., 0.4 per cent pyronin G. B.D.H., 0.25 per cent phenol in water) or with pyronin alone (1 per cent pyronin G. B.D.H., 0.25 per cent phenol in water); when fixed in acetic alcohol the cultures were stained with methyl-green pyronin.

The cultures for phase contrast observation were taken from the incubator as required and immediately placed in the warm chamber at 37°C. Cooke, Troughton and Simms' phase contrast equipment was used and illumination was by a 250 W "Mercra" high pressure mercury arc. A Cooke, Troughton and Simms green filter was used and the cultures were only illuminated for brief periods insufficient to cause damage to the cells. Photographs were made with a Cooke, Troughton and Simms' fixed length eyepiece camera on $3\frac{1}{4}'' \times 4\frac{1}{4}''$ Ilford Rapid Process Panchromatic plates. Under these conditions and with a × 95 oil immersion objective, a × 10 eyepiece and a 0.30 neutral density filter (reducing incident light to $\frac{1}{2}$), in addition to the green filter, a 1-second exposure was adequate. For visual observation further neutral filters were inserted so that the intensity of illumination was the minimum needed to reveal essential detail.

RESULTS

Observations on stained preparations. In the experiments of Series 1, in which tissue was grown in a medium containing additional cobalt, no specific cytological effect was observed up to a concentration of cobalt rather greater than 1×10^{-5} g/ml when outgrowth was completely inhibited. There was, however, a threshold concentration of cobalt below which outgrowth was abundant and mitoses normal, and above which outgrowth was slight and mitoses rare. Consequently these experiments could not demonstrate whether cobalt had any action on the mitotic process itself.

In the first experiments of Series 2, horseshoe cultures were used and were treated with cobalt as described above, after which they were incubated for a further 24 hours and then fixed and stained with May-Grünwald-Giemsa (6) in order to study any possible disturbance of the normal distribution of deoxyribosenucleoprotein (DRNP) and ribosenucleoprotein (RNP). Concentrations of cobalt in the medium $< 3 \times 10^{-6}$ g/ml were without obvious effect, and concentrations $> 3 \times 10^{-5}$ g/ml were grossly toxic, both outgrowth and mitosis being completely inhibited while the remaining cells appeared shrunken and distorted. At intermediate concentrations of 1 to 1.5×10^{-5}

g/ml, however, outgrowth and mitosis were not greatly reduced and a variety of cytological effects was observed. The most obvious abnormality was the irregular nuclear shape, and especially in the horseshoe cultures deeply lobed and annular forms were fairly frequent (Fig. 8). Such effects are not specific, however, and may be produced by various agents. A more interesting phenomenon was the presence of one and, more rarely, two spherical bodies in the cytoplasm of telophase and interphase cells (Figs. 9, 8). These bodies were stained dark blue by the May-Grünwald-Giemsa method indicating that they contained RNP, and their appearance suggested that at one time they had been nuclear nucleoli. As the metaphase and anaphase chromosomes were also stained dark-blue, in preparations made by this technique, it was difficult to find how these RNP bodies had arrived in the cytoplasm. In cultures fixed in Maximow's fluid and stained with Ehrlich's haematoxylin, although there was no colour differentiation for the two types of nucleoprotein, the cytological detail was very clear and in addition to the cytoplasmic bodies observed in telophase a similar spherical or oval structure was frequently seen at one extremity of the metaphase plate or as a bridge between the chromosome groups in anaphase. It now seemed almost certain that the cobalt had caused nucleoli to persist throughout mitosis.

These observations were repeated on standard hanging drop cultures. It was decided to change the type of culture because occasionally in both

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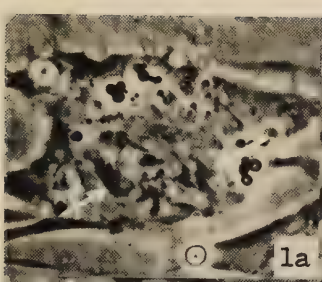
Figs. 1 and 2. Serial photographs of two living fibroblasts in mitosis showing nucleolar persistence caused by added cobalt chloride. Chick heart × 950. Phase contrast. Persistent nucleoli are indicated by arrows.

Fig. 1 a. Very late prophase—two nucleoli.	0 min.
Fig. 1 b. Methaphase—one nucleolus at each side of metaphase plate.	4 ,,
Fig. 1 c. Metaphase.	10 ,,
Fig. 1 d. Early anaphase—nucleoli forming bridges between separating chromosome groups.	13 ,,
Fig. 1 e. Late anaphase.	21 ,,
Fig. 1 f. Telophase—both nucleoli have gone to right hand daughter cell.	23 ,,
Fig. 1 g. Telophase—new nucleoli in daughter nuclei.	40 ,,
Fig. 1 h. Same.	55 ,,
Fig. 1 i. Late telophase with daughter nuclei.	63 ,,
Fig. 1 j. Same cells fixed in methyl alcohol and stained with methyl-green pyronin immediately after the taking of photograph 1 i. The daughter nuclei have each formed two new nucleoli and the two persistent nucleoli lie in the cytoplasm of the right hand cell.	
Fig. 2 a. Metaphase with one persistent nucleolus at side of metaphase plate.	0 min.
Fig. 2 b. Early anaphase—nucleolus forming a bridge.	2 ,,
Fig. 2 c. Late anaphase—nucleolus being torn into two.	5 ,,
Fig. 2 d. Early telophase—chromosome groups now separate, each with a portion of the persistent nucleolus.	7 ,,
Fig. 2 e. Telophase—each daughter cell contains a portion of the original persistent nucleolus.	12 ,,
Fig. 2 f. Late telophase, new nucleoli have formed in the daughter nuclei.	45 ,,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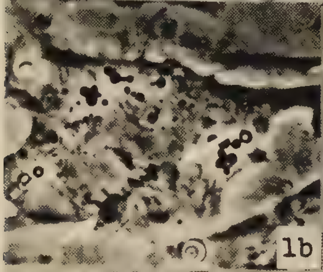
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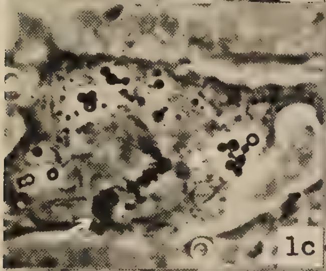
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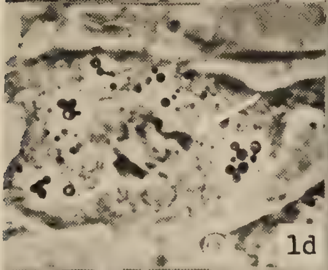
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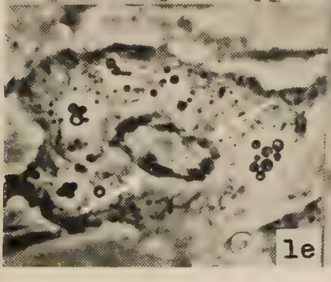
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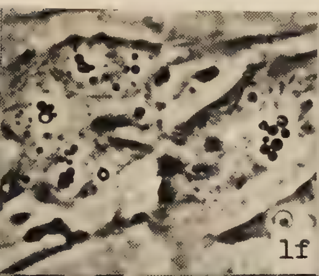
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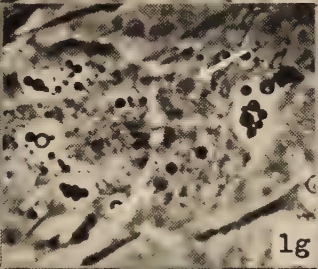
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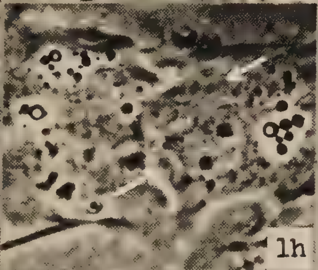
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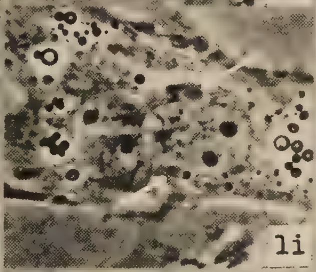
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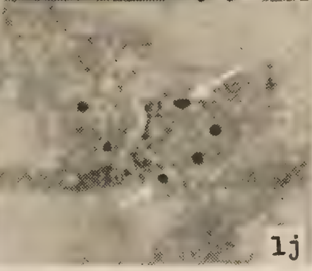
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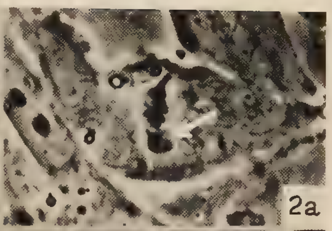
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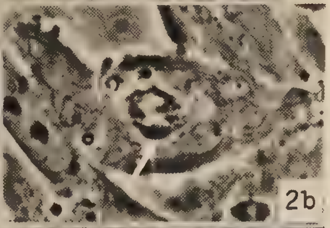
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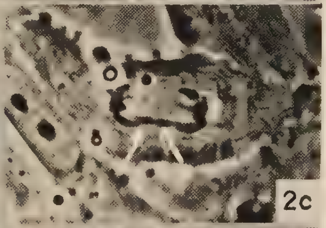
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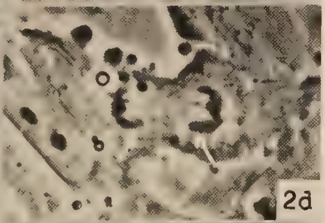
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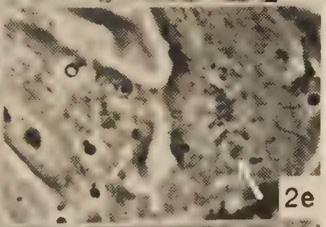
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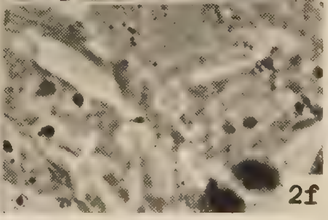
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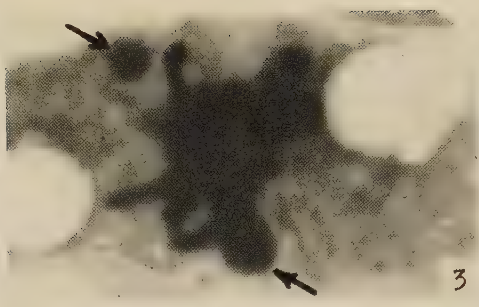

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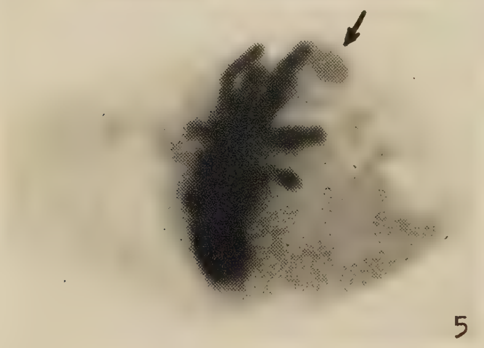

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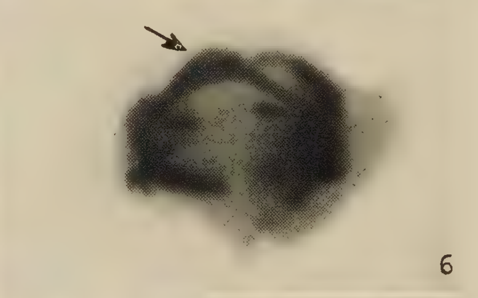

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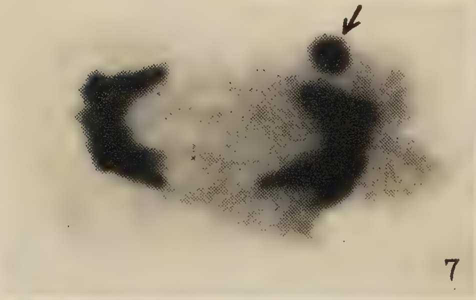

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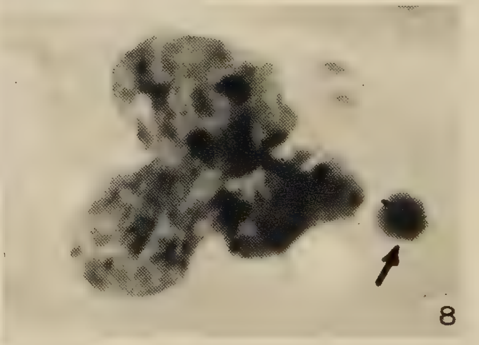

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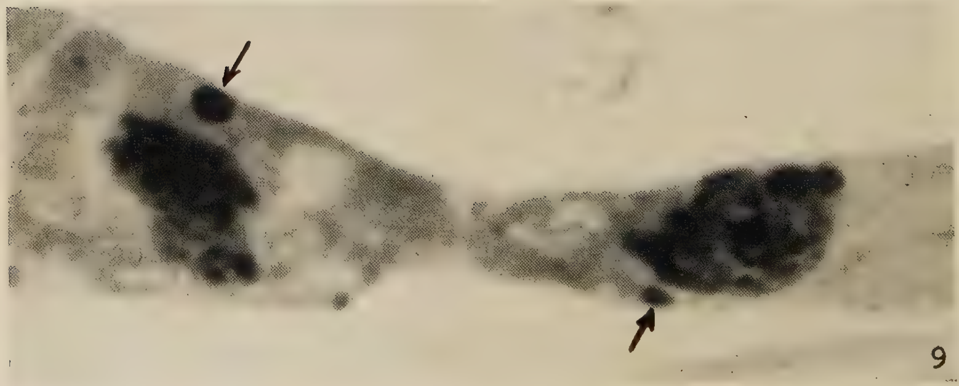

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untreated and Tyrode-serum-treated controls of the horseshoe type, a few obviously abnormal cells were found; in such cells, however, the nucleoli did not persist throughout mitosis. For these later experiments methyl-green pyronin staining was adopted after fixation in methanol or acetic alcohol in order to obtain a colour differentiation between the chromosomes and the persistent nucleoli in meta- and anaphase. In such preparations persistent nucleoli were conspicuous in cobalt-treated cultures, as bright red drops at either side of the green or bluish-green metaphase plate and bridging the green chromosome groups in anaphase. In telophase, it was observed that in spite of the presence of persistent nucleoli in the cytoplasm, fresh nucleoli were formed in the daughter nuclei.

The presence of persistent nucleoli in the later stages of mitosis was variable in cultures fixed 24 hours after treatment with cobalt, and it was suspected that this was not the optimum period for studying the effect. Accordingly cultures were fixed at 2-hourly intervals up to 24 hours after the addition of Tyrode-serum and Tyrode-serum-cobalt. It was then found that mitosis ceased until about the 3rd hour in the cultures treated with Tyrode-serum and until about the 6th hour in those treated with Tyrode-serum-containing cobalt. After this interval the numbers of cells entering mitosis rose to the usual levels in both experimental and control cultures. The lag period was partly due to the cooling of the cultures during the handling and partly to the addition of Tyrode-serum; the greater lag in those treated with cobalt was presumably due to the metal. Once the Tyrode-serum-treated cultures had recovered from this latent period, no mitotic abnormalities of importance were observed. In those to which Tyrode-serum-cobalt had been added, mitotic cells with persistent nucleoli were most numerous 10–14 hours after the addition; after 24 hours, although the abnormality was still present, it was less frequent and did not appear in every culture. Consequently the 10–14 hour period was selected for further study of the effect.

Figs. 3–9. Different chick heart fibroblasts from a single culture to which cobalt chloride had been added showing persistent nucleoli. Fixed in Maximow's fluid and stained with Ehrlich's haematoxylin. × 3200. Persistent nucleoli are shown by arrows.

Fig. 3. Early metaphase, showing one free persistent nucleolus and one in association with the chromosomes.
Fig. 4. Metaphase with two free persistent nucleoli.
Fig. 5. Metaphase with one attached persistent nucleolus.
Fig. 6. Anaphase with persistent nucleolus forming a bridge between separating chromosome groups.
Fig. 7. Anaphase with persistent nucleolus accompanying one chromosome group only.
Fig. 8. Interphase cell showing lobulated nucleus and extranuclear nucleolus.
Fig. 9. Telophase showing daughter cells, each with one cytoplasmic persistent nucleolus and fresh nucleoli in the daughter nuclei.

In an attempt to reduce the initial lag in mitosis due to chilling, a batch of cultures was treated with Tyrode-serum at 37°C inside the opened incubator; the temperature during this process probably fell to about 30°C at the culture. These cultures were then fixed at 15, 30, 45, 60 and 90 minutes after the addition. The results showed that cells already in mitosis when the Tyrode-serum or Tyrode-serum-cobalt was added completed division normally, but after 45 minutes no further mitotic cells could be seen in either set of cultures. No persistent nucleoli were found in any of the cultures treated with cobalt and fixed within the range of 15 to 90 minutes after treatment. Thus it would seem that the main effect was produced at the end of the intermitotic period of 10–12 hours after the addition of cobalt.

Warm stage observations on living cells. In order to follow the process of nucleolar persistence more closely, standard hanging drop cultures were treated with Tyrode-serum-cobalt and then placed under a microscope in a warm chamber for direct observation with phase contrast illumination. The results confirmed the conclusions drawn from a study of fixed and stained preparations (compare Figs. 1 and 2 with Figs. 3–9).

The stages of normal mitosis in cultures of this type as seen by phase contrast microscopy have been clearly described by Hughes and Fell (5), and in many observations it has been found that the nucleolus disappears late in prophase almost simultaneously with the nuclear membrane. In the living cobalt-treated cultures, on the other hand, the nucleoli remained after the nuclear membrane had vanished and were clearly seen in association with the chromosomes during the radial stage of metaphase which follows (Figs. 1 a, 3). When the metaphase plate was formed the nucleolus lay at one side of the plate and if two nucleoli persisted they were situated at opposite sides (Figs. 2 a, 5, and 1 b–1 c).

At anaphase the nucleolus formed a bridge between the separating chromosomes (Figs. 1 d–1 e, 2 b–2 c, 6); either it went wholly to one or other daughter cell or it adhered to both groups and was stretched and torn into two, one piece accompanying each group (Figs. 1 f–1 j, 7, and 2 c–2 f, 9). At telophase the persistent nucleolus was excluded by the new nuclear membrane and thereafter lay free in the cytoplasm where it underwent a slow random movement similar to that of the mitochondria. Nevertheless, other nucleoli were always formed in the nuclei of the daughter cells at the usual stage of reconstruction. In general the process was fairly constant and regular but slight variations were noticed. For example in one cell at the radial stage of metaphase the nucleolus broke loose from the chromosomes and did not immediately reach the metaphase plate; approximately 1 hour elapsed before it gradually migrated to the plate and immediately afterwards a normal anaphase took place. Metaphases in fixed preparations occasionally show the same phenomenon (Fig. 4).

The effect of nucleolar persistence on the distribution of the nucleic acids. Apart from the persistence of the RNP in the discrete nucleolar body, mitotic and interphase cells in cobalt-treated cultures showed no obvious disturbance of the normal distribution of RNP and DRNP. Mitotic cells usually completed division within the ordinary period, and this suggests that the nucleolar RNP is not essential to the mitotic process. Staining by the May-Grünwald-Giemsa method, by methyl-green pyronin, and by pyronin alone, demonstrated that the chromosomes had an RNP component during prophase and metaphase as in normal cells (6, 7) in spite of the unavailability of the nucleolar RNP. Jacobson and Webb (6) have described a deeply staining basophilic area between the two groups of chromosomes at anaphase, which they attribute to transfer of RNP from the chromosomes into the cytoplasm. As the intensity of basophilic staining varies widely in anaphase cells, in normal untreated cultures, both in the area between the chromosome groups and in the remainder of the cytoplasm, it was not possible by purely visual methods to assess local variations in the concentration of RNP. Therefore it is not known if cobalt affects the cytoplasmic distribution of RNP at this stage of mitosis.

DISCUSSION

Although the older literature contains references to the nucleoli of plant cells persisting throughout mitosis, to the author's knowledge this occurrence has not been described in animal cells. That it should be possible to cause this nucleolar persistence by using a concentration of cobalt well below the toxic level is of interest for two reasons: firstly because it is the nucleolar RNP alone which seems to be affected, and secondly because the effect is apparently specific for cobalt; neither nickel, zinc, chromium nor manganese in sublethal concentration produce the same effect (other metals are being tested).[1] Whether this action of cobalt is due to its combination with nucleolar RNP so that it acts as a fixative for this material, or whether it is due to the inhibition of a nucleolus-dispersing enzyme remains to be seen. It is hoped that further investigations may elucidate this point and in doing so shed light on the events which normally lead to the disappearance of the nucleolus during late prophase.

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[1] In addition to the metals mentioned, neither iron nor beryllium in sublethal concentration has been found to cause nucleolar persistence under the same experimental conditions.

SUMMARY

1. Embryonic heart and frontal bone were explanted into normal culture medium to which cobalt (as chloride) had been added. There was a threshold concentration of the metal at which outgrowth was almost normal and no specific cytological effects were seen. Slightly above this level, growth was completely inhibited.

2. When cobalt was added to cultures of embryonic chick heart during their active growth phase, the nucleoli persisted from prophase to telophase in a high proportion of the mitoses. The effect was greatest 10–14 hours after the addition of the cobalt. The sequence of events in living cells is described and the possible significance of the phenomenon is discussed.

During the course of these experiments the author has derived great benefit from discussions with Dr. Honor B. Fell, Director of this laboratory, and with his other colleagues, and here acknowledges his debt to them.

In the preliminary experiments the tissue cultures were made by Mr. L. J. King and in the main experiments by Miss J. Rawlinson. Miss J. Such stained the cultures. To these assistants the author expresses his thanks for their careful and conscientious work.

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GROWTH FACTORS IN THE DIGESTION APPARATUS OF RAT[1]

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The presence of mitosis-stimulating substances in homologous tissue suspensions has been demonstrated in the outer orbital gland (9), in the epidermis (11) and in the liver (12) of rat.

Regeneration processes in the alimentary canal and particularly in the stomach attract special attention owing to the high incidence of disease in these organs. Since the stomach of the rat consists of a forestomach, whose structure resembles that of the esophagus, and a glandular stomach which corresponds to the ordinary stomach of mammals, the effect of suspensions of these two portions was investigated separately. As the different parts of the alimentary canal are closely related to another developmentally, the effect of suspensions of the small and large intestine was investigated.

In a previous study (13) we found that a continuous physiological cell division takes place in the stomach of the rat. About one per thousand of the cells both in the forestomach and the glandular stomach of normal adult animals are in mitosis.

METHODS

As experimental animals 4–6-week-old rats of the Wistar strain were used. The effect of the different tissue suspensions on the mitotic ratio in the epithelium of the forestomach and the glandular stomach was investigated.

The tissue suspensions were prepared from newborn rats. Immediately after decapitation, a fixed quantity of the tissue whose effect was to be tested was weighed and mashed in a mortar. Saline solution was added so that 1 ml of the suspension always corresponded to a fixed quantity of tissue. The suspensions were injected intraperitoneally at the same time of day. The treatment of the rats was in every respect the same. They were decapitated at the same time of day 24 and 48 hours after application. In this way errors due to the diurnal rhythm are avoided. Such a rhythm has been shown to exist in the occurrence of mitoses in the stomach (6) and in the small intestine (5) of the rat. The stomach was immediately removed without being opened. It was rinsed with a 10 per cent formaline solution, and filled with the same solution. It was then ligated at the duodenum and esophagus, and placed

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in a 10 per cent formaline solution. By this method good sections were obtained. These were 5 μ thick and stained with hemaleum-eosin. The mitotic index was determined per thousand cells in the forestomach and the glandular stomach by the same method as before (13).

RESULTS

The first experimental series comprised injection of a suspension of the forestomach and of the glandular stomach into 4-week-old rats. The doses were 2, 1, 0.5, 0.25, 0.1 and 0.05 mg of tissue. Since the mitosis-stimulating agent in the outer orbital gland has proved very thermostabile (10) suspensions heated for 15 minutes at 100° C were also given.

Suspensions of forestomach. For this experiment 45 rats weighing 55 to 95 gm were used. In the two different temperature groups a distinct increase of the mitotic value was obtained in the forestomach, while in the glandular stomach the ratio hardly diverged at all from the values obtained for the controls (Table I). The effect was strongest 48 hours after application (Table II). The highest mitotic value in all our experiments, 24 per thousand, was obtained with 0.5 mg. The effect was smallest with the lowest dose. There was considerable individual variation.

Suspensions of glandular stomach. This group comprised 46 rats whose weight varied between 45 and 85 gm. With nonheated suspensions high mitotic indices were obtained both in the forestomach and in the glandular stomach (Table I). The effect was almost equally great 24 and 48 hours after injection (Table II). The highest mitotic index, 19 per thousand, was obtained in the glandular stomach of a rat 24 hours after application of 0.5 mg of tissue suspensions. In the same group, however, mitotic indices as high as 18 per thousand were obtained in the forestomach both 24 and 48 hours after injection. After heating the effect was definitely weaker. Values that clearly exceeded those for the controls were only obtained in the forestomach 48 hours after injection.

The second experimental series comprised injections of a suspension of the stomach, the small intestine and the large intestine. Since in the first experimental series 0.5 mg had produced the highest stimulation, this dose alone was now used. The small intestine suspension was derived from 3 cm of the duodenum immediately distal to the pylorus. The large intestine suspension was made from equal quantities of the ascending and the transverse colon. The stomach suspension was made from equal quantities of forestomach and glandular stomach. The suspensions were tested on 23 4-week-old rats.

TABLE I

Mitotic index in the stomach epithelium of 4-week-old rats after intraperitoneal injections of tissue suspensions of forestomach and glandular stomach.

Tissue suspensions		Number of rats	Mitotic index	
			Forestomach	Glandular stomach
20°C	Forestomach	23	6.57±0.84	5.43±0.54
	Glandular stomach . . .	23	7.30±0.56	8.59±0.70
100°C	Forestomach	22	4.68±0.37	6.00±0.38
	Glandular stomach . . .	23	3.87±0.32	5.63±0.36
	Controls	8	1.88±0.22	4.25±0.27

TABLE II

Effects of time of influence of intraperitoneally injected suspensions of forestomach and glandular stomach on the mitotic index in the stomach epithelium of 4-week-old rats.

Tissue suspensions		Time of influence, hours	Number of rats	Mitotic index	
				Forestomach	Glandular stomach
20°C	Forestomach	24	12	2.42±0.30	3.75±0.36
	,, ...	48	11	11.09±1.18	7.27±0.94
	Glandular stomach . .	24	11	6.45±0.48	9.00±1.26
	,, ,, . .	48	12	8.08±0.68	7.83±0.70
100°C	Forestomach	24	12	3.92±0.40	5.17±0.44
	,, ...	48	10	5.60±0.64	7.00±0.62
	Glandular stomach . .	24	11	2.55±0.24	5.45±0.48
	,, ,, . .	48	12	5.08±0.48	5.83±0.54
	Controls		8	1.88±0.22	4.25±0.27

Stomach suspension. In the forestomach no certain stimulation of the mitotic activity was observed (Table III). With heated suspension an increase was obtained. In the glandular stomach the mitotic index was clearly increased after injection both of usual and of heated suspension.

Small intestine suspension. The mitotic activity was increased both in the forestomach and in the glandular stomach. With heated suspension the mitotic index was somewhat increased in the forestomach and clearly increased in the glandular stomach (Table III).

TABLE III

Mitotic index in the stomach epithelium of 4-week-old rats after intraperitoneal injections of tissue suspensions of stomach, small and large intestine.

Tissue suspension		Number of rats	Mitotic index	
			Forestomach	Glandular stomach
20°C	Stomach	4	3.25 ± 0.13	8.75 ± 1.14
	Small intestine	3	6.67 ± 1.42	6.00 ± 2.00
	Large intestine	4	4.50 ± 0.40	7.50 ± 0.61
100°C	Stomach	4	5.75 ± 1.09	9.50 ± 0.60
	Small intestine	4	4.50 ± 0.67	9.25 ± 0.33
	Large intestine	4	6.50 ± 0.82	10.25 ± 0.84
	Controls	3	2.00 ± 0	4.67 ± 0.40

TABLE IV

Mitotic index in the stomach epithelium of 6-week-old rats after intraperitoneal injections of tissue suspensions of stomach and skin.

Tissue suspensions	Number of rats	Mitotic index	
		Forestomach	Glandular stomach
Stomach	4	3.00 ± 0.38	4.75 ± 0.82
Skin	4	1.50 ± 0.27	2.50 ± 0.47
Saline solution	5	2.60 ± 0.59	2.60 ± 0.30
Controls	3	2.20 ± 0.35	4.57 ± 0.33

Large intestine suspension. A mitosis-stimulating effect was obtained both in the forestomach and in the glandular stomach (Table III). After heating the positive effect further increased in both portions of the stomach.

In the third experimental series a comparative experiment was performed by testing suspensions of both parts of the stomach and of the skin on thirteen 6-week-old rats. Skin suspension derived from newborn rats was used; such a suspension has been shown to have a mitosis-stimulating effect on the epidermis (11) of the white rat. The similarity between the epidermis and the epithelium of the forestomach was another reason for the choice of this particular tissue. As a control, saline solution was given to rats of the same age. The suspensions were prepared as before but were not heated.

Stomach suspension. In the forestomach the mitotic index increased after 24 hours but had returned to normal after 48 hours. In the glandular stomach the index was normal (Table IV), as was also the case in the epidermis.

Skin suspension. Neither in the forestomach nor in the glandular stomach could any mitosis-stimulating effect be observed (Table IV). The mitotic ratio in the epidermis, however, was clearly affected by the skin suspension. The mitotic index was 5.12 ± 0.47 per thousand 24 hours after the injection and 1.8 ± 0.30 per thousand after 48 hours, which value correspond to normal indices (11). The mitotic index in the epidermis was not influenced by suspensions of stomach or intestine.

Saline solution. No effect could be observed in the forestomach or the glandular stomach. Neither were the mitotic indices in the skin increased.

DISCUSSION

The experiments showed that tissue suspensions both of the forestomach and the glandular stomach, of small intestine and large intestine have a mitosis-stimulating effect on the epithelium of the forestomach and of the glandular stomach of the white rat. The mitosis-stimulating agent or agents were for the most part active after treatment for 15 minutes at 100° C, thus resembling the mitosis-stimulating agent in the orbital gland of rat (10).

The mitoses were normal as were those induced in a similar way in the outer orbital gland (9) and the liver (12). The location of the mitoses both in the forestomach and in the glandular stomach was the same as that in normal animals (13). Only mucous cells were seen in mitosis after application of tissue suspensions.

Whether the effect obtained is of an enterogastron (2, 3, 4) or robudene (1, 7, 8) type is hard to decide on the basis of these experiments. These extracts were likewise derived from small intestine and stomach tissue and proved to exert a beneficial effect on secretion and on the healing of gastric ulcers. No exact investigations have been made with these agents with regard to their effect on mitosis. No direct comparison can therefore be made.

The mitosis stimulating agents are supposed to be closely related to the induction agents and may perhaps develop from these (14). The different parts of the alimentary canal are closely related developmentally and it is therefore possible that the corresponding growth hormones resemble one another with regard to their biological effect. This would explain the obvious mitosis-stimulating effect of the small intestine and the large intestine suspensions on both the forestomach and the glandular stomach.

SUMMARY

The mitosis-stimulating effect of tissue suspensions of different portions of the alimentary canal on the stomach epithelium of the rat was investigated. Suspensions of forestomach, glandular stomach, small intestine and large intestine were found to possess a mitosis-stimulating effect upon the epithelium of the stomach. Suspensions of forestomach and intestine were active even after heating to 100° C for 15 minutes.

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SYNERGISM OF NUCLEOPROTEIN AND DIALYSATE GROWTH FACTORS IN CHICK EMBRYO EXTRACT[1]

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Investigations of growth-promoting agents for cells in tissue culture have for many years stressed the role of protein factors, especially those occurring in chick embryo extract. Studies directed toward isolation of the active agents were begun by Baker and Carrel (1, 2) and continued by Fischer and associates (6, 8, 9). Fischer and Astrup (9) eventually succeeded in preparing a nucleoprotein fraction from chick embryos by the method of Hammarsten (11) and found that this material stimulated vigorous proliferation of fibroblasts in tissue culture. Later Davidson and Waymouth (4) obtained a similar result but did not purify the final product further. Recently Kutsky (15) has isolated a nucleoprotein fraction of high activity from chick embryos by selective precipitation with streptomycin from saline extracts. Evidence from electrophoresis and direct analysis indicates that growth stimulation may be associated with one or more ribonucleoproteins in this fraction.

Other studies on the growth-promoting properties of embryo extract have emphasized the role of crystalloidal factors occurring in ultrafiltrates or dialysates (7, 8, 10, 13, 14, 20, 24). As one means of assay Fischer (7) introduced the use of dialyzed culture media and showed that the addition of dialysates from chick embryo extract produced conspicuous out-growth of fibroblasts. Recently Harris (12) has modified and extended these assay procedures. It has not yet been possible to isolate the active growth factors from dialysates, although some progress has been reported in the purification of material obtained from calf embryos (10) and from chick embryos (13, 14).

The parallel lines of investigation outlined above have a meeting point in Fischer's concept (8) of an interaction between non-dialyzable components ("embryonin") and dialyzable components ("accessory growth factors") in chick embryo extract. In the present investigation we have explored this possibility and established a synergism between active growth-promoting principles in the nucleoprotein (15) and barium-soluble components respectively. Nucleoprotein (15) and barium-soluble dialysate (14) fractions were prepared for this purpose and added to cultures of chick heart fibroblasts. The basal assay media were dialyzed, thus permitting study of the separate effects of the two fractions as well as their action in combination. Finally, data have been obtained which show that synthetic supplements also synergize with the nucleoprotein fraction and may substitute at least in part for the naturally-occurring dialysate factors.

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MATERIALS AND METHODS

Preparation of dialysate fractions. A detailed description of the procedures used may be found in preceding papers (13, 14). In essence, this involves homogenizing 12-day chick embryos in ethanol to a final concentration of 70 per cent, extraction of the clear supernatant with chloroform, dialysis, and evaporation of the pooled dialysates. The residual non-dialyzable fraction is discarded. The crude dialysate in aqueous solution is then treated with $BaCl_2$ at pH 8.2. An inert precipitate is discarded. The active factors remaining in solution may be further subdivided by adjusting the concentration of ethanol to 80 per cent (14). In the present experiments, however, the latter two subfractions were combined again and will be referred to simply as the *barium-soluble dialysate fraction.* For assay purposes, Ba^{++} was removed from this material by adding a slight excess of Na_2SO_4, and sterilization effected by passage through a sintered glass filter (ultrafine).

Preparation of nucleoprotein fraction. A full account of the method used has been given in a recent paper (15) which may be consulted for details. The isolation procedure is carried out in the cold and involves homogenizing 12-day embryos in borate solution, followed by precipitation with streptomycin from the supernatant. The residual solution is discarded, the precipitate reconstituted in phosphate, dialyzed and finally re-precipitated with streptomycin. After a second reconstitution in phosphate and thorough dialysis to remove streptomycin the preparation is centrifuged at 105,000 Xg for an hour. The transparent middle fraction is removed, dialyzed against Gey's solution, and centrifuged again at 105,000 Xg for an hour. The final supernatant is clear, colorless, viscous, and displays a typical nucleoprotein absorption spectrum in the ultraviolet.

Assay procedures. Cultures were made in Carrel flasks using explants of 12–15 day chick heart. The basal assay medium consisted of dialyzed chicken plasma clotted by minimal amounts of dialyzed chick embryo extract (0.02 ml. per flask), plus a supernatant of dilute dialyzed horse serum (40 per cent horse serum, 60 per cent Gey's saline). Embryo extract was omitted from the supernatant fluid. The medium contained 0.15 per cent D-glucose, 0.002 per cent phenol red, 100 units/ml penicillin G (Merck), and 50 units/ml dihydrostreptomycin (Pfizer). Test fractions were made up in Gey's solution and incorporated at appropriate levels in the assay medium. The cultures were incubated at 38°C. for 10 days with fluid changes after 4 and 7 days.

respectively. In addition to microscopic observation during this period, areal increases were measured with a projectoscope and planimeter. In some instances lactic acid in the supernatant fluids were determined spectrophotometrically by the method of Barker and Summerson (3). Details of culture methods, including the preparation and use of dialyzed media, may be found in a preceding publication (12).

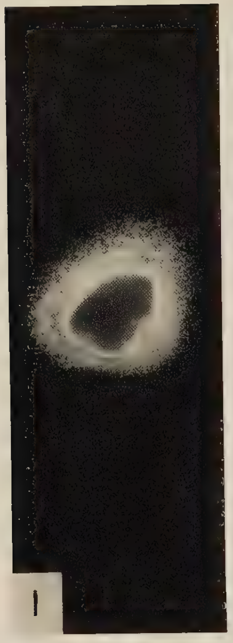
RESULTS

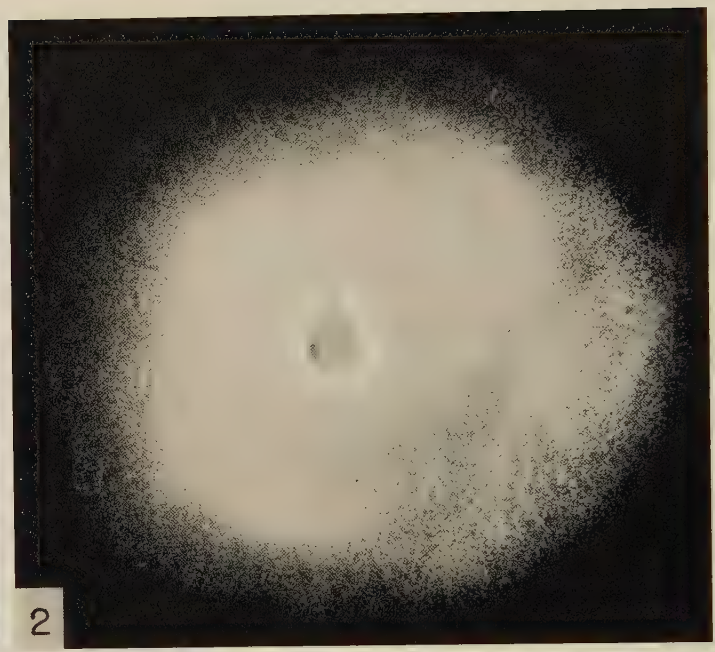
Interaction of nucleoprotein and dialysate fractions

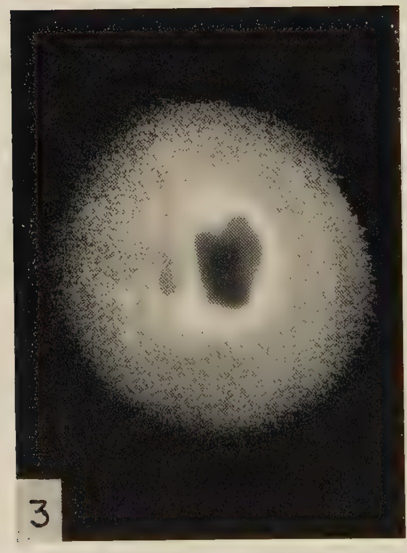
Plan of experiments. Four types of cultures were established in the dialyzed plasma-serum assay medium. An initial group without supplementation served as controls. Barium-soluble dialysate was added to a second series and nucleoprotein fraction to a third. The fourth group of cultures received both dialysate and nucleoprotein fractions. Final concentrations in the assay medium were 2.1 mg/ml dialysate and 0.6 mg/ml nucleoprotein fraction.

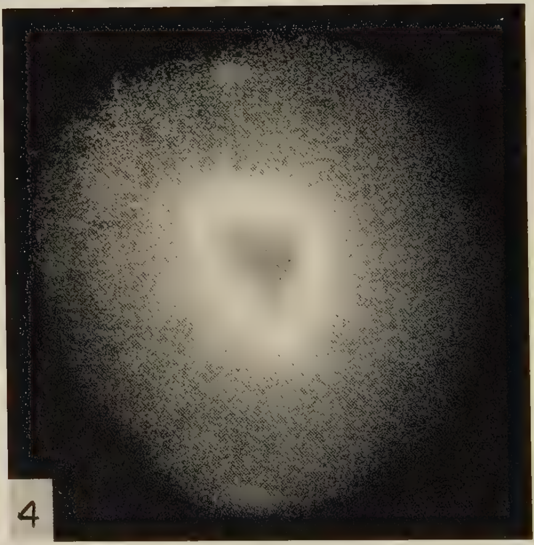
Gross changes. Differences in pattern of outgrowth between the several experimental series could be detected as early as two days after explantation and were clear-cut at the end of the experimental period (10 days). In unsupplemented basal medium outgrowths were characteristically confined to a narrow fringe of cells bordering the original explant (Fig. 1). The marginal areas were appreciably larger in cultures supplemented with nucleoprotein fraction. These outgrowths presented a dense, compact appearance with sharply defined edges (Fig. 3). The addition of barium-soluble dialysate alone produced large increases in surface area. In these cultures the outgrowth zone was characteristically thin, nearly transparent (Fig. 4). Cultures supplemented with both nucleoprotein and dialysate fractions were characterized by a dense, even pattern of out-growth (Fig. 2) and of the experimental series in question most closely duplicated the effects of fresh whole embryo extract.

Table I presents a comparison in summary form of the changes in surface area. It is apparent that the nucleoprotein and dialysate fractions have less effect added singly than in combination. These figures, moreover, do not reflect the striking differences in density of outgrowth between cultures containing both fractions and those containing dialysate only. That these differences are correlated with a disparity in cell populations is further suggested by analyses for lactic acid in the supernatants. The resulting data (Table I) while not strictly paralleling the changes in surface area nevertheless show a similar trend.









All photomicrographs taken with the assistance of Mr. Victor Duran.

Figs. 1–4. Outgrowth of chick heart fibroblasts at 10 days in experimental cultures. × 5.6.
Fig. 1. Unsupplemented basal medium.
Fig. 2. Basal medium supplemented with both nucleoprotein and barium-soluble dialysate fractions.
Fig. 3. Basal medium supplemented with nucleoprotein fraction only.
Fig. 4. Basal medium supplemented with barium-soluble dialysate only.

TABLE I

Effects of nucleoprotein and barium-soluble dialysate fractions of embryo extract on chick heart fibroblasts

Fraction added	Concentration in medium mg/ml	Lactic acid[2] in supernatants at 8 days, micrograms/ml	Areal increase[3] at 11 days, mm²
None (basal medium[1])	—	268	19.9 ± 4.5
Nucleoprotein	0.6	488	39.6 ± 4.2
Barium-soluble dialysate . . .	2.1	526	82.4 ± 10.0
Nucleoprotein plus dialysate .	each as above	803	118.0 ± 4.3

Microscopic changes. After a few days a characteristically different cell picture was observed in each of the four experimental series. Out-growths in unsupplemented medium consisted of a sparse population of shrunken cells in an open, net-like arrangement. Inclusions were prominent after a few days (Fig. 5). In cultures containing added nucleoprotein fraction the cells were closely massed together. These became refractile and heavily loaded with vacuoles so that the outgrowth zone soon assumed an opaque appearance (Fig. 6). In contrast the peripheral outgrowth in cultures containing barium-soluble dialysate consisted of triangular or spindle-shaped elements, without conspicuous inclusions and non-refractile in appearance (Fig. 7). Here the cells became more widely spaced as the marginal zone expanded. The optimal cell picture was observed in outgrowth zones from cultures containing both nucleoprotein and dialysate fractions. These cells were expanded in form, non-refractile, and typically were arranged in dense "swirls" or were radially oriented with respect to the original explant (Fig. 8). The regularity of this pattern, the uniform density of cell population, and the relative absence of inclusions in peripheral cells were reminiscent of cultures containing fresh whole embryo extract. Microscopic observation thus confirms the interaction of nucleoprotein and dialysate fractions to give a more complete effect than obtained with either fraction alone.

Interaction of nucleoprotein and synthetic supplements

Morgan-Parker Mixture No. 199. Several workers have attempted to devise a complete nutrient for animal cells *in vitro* using known crystalloidal growth

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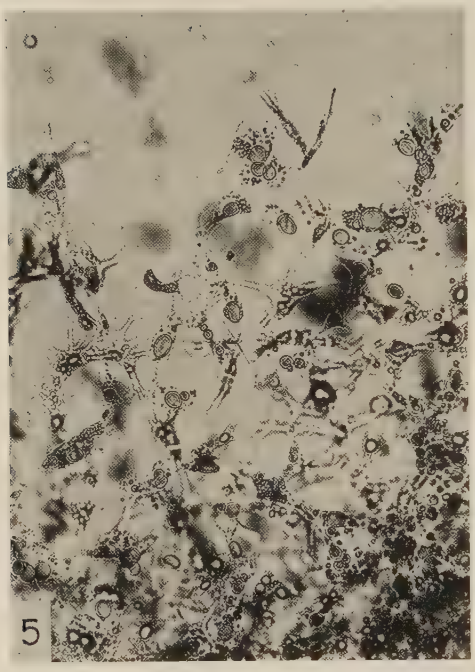
[1] *Basal medium:* dialyzed chicken plasma + supernatant 40 per cent dialyzed horse serum, 60 per cent Gey's solution.

[2] Average values for duplicate determinations on pooled supernatants from six cultures.

[3] Mean value and standard error (21).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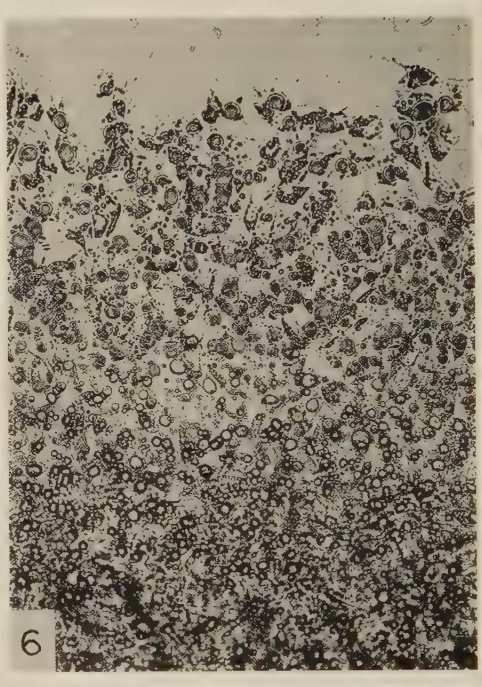
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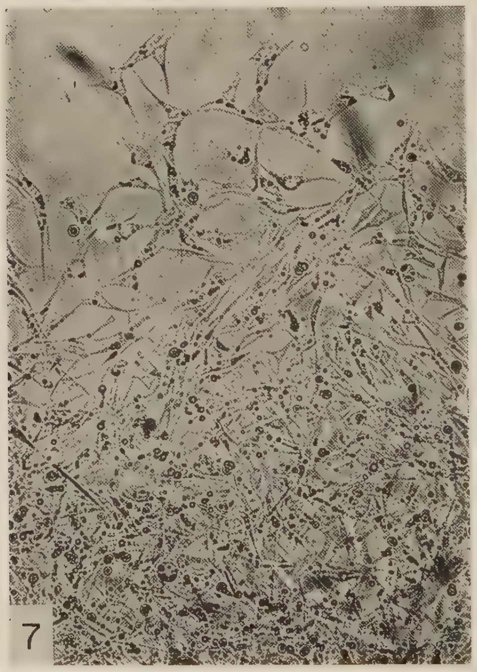
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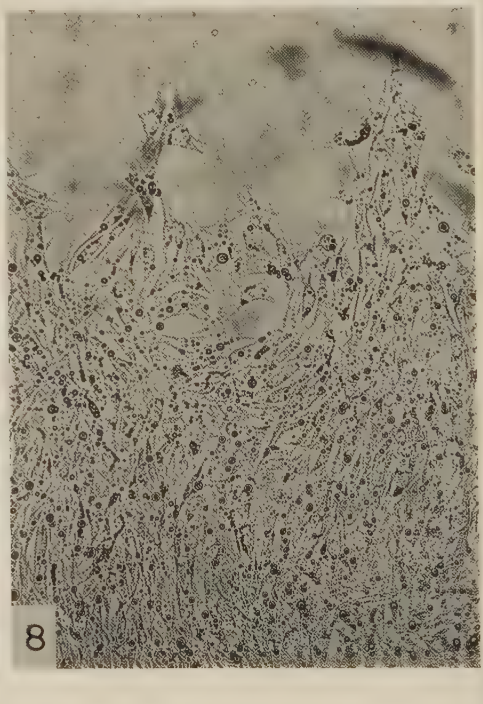
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factors (16, 17, 18, 19, 22, 23). The most widely used of these nutrients is Medium No. 199 as first described by Morgan, Morton, and Parker (16). Although Mixture No. 199 is not adequate for growth and permits survival for limited periods only (5) it has proved useful for virus culture and other studies. It seemed of interest to explore the possibility that this defined nutrient might substitute for the naturally occurring dialysate factors in the synergism noted with the nucleoprotein fraction. Accordingly experiments were performed using aliquots of Medium No. 199 prepared commercially by Difco Corp., Detroit, Michigan. Figures 9–12 and Table II summarize the results of this investigation. In media containing 25 per cent Mixture No. 199 alone the outgrowths are somewhat larger than controls (Figs. 1, 9) but are markedly deficient in microscopic appearance. Cells in the marginal zone are packed irregularly together and exhibit a prominent vacuolation (Fig. 11). On the other hand a clear-cut interaction is observed when both nucleoprotein and Mixture 199 supplements are added to the Medium. The resulting outgrowths (Fig. 10) are much larger than cultures containing either nutrient singly (Figs. 3, 9). The microscopic picture (Fig. 12) is less favourable than that seen in cultures containing both dialysate and nucleoprotein fractions (Fig. 8). Clear evidence of interaction is seen, however, if the appearance of cells in media containing both nucleoprotein and 199 (Fig. 12) is compared with the effects produced by each supplement when added alone (Figs. 6, 11).

Table II documents the synergism observed between Mixture No. 199 and the nucleoprotein fraction. It may be seen that both in terms of surface area and production of lactic acid the experimental cultures receiving both supplements were far in advance of the others.

Other supplements. In view of the interaction of glutathione and cystine with one subfraction of the barium-soluble dialysate (14), experiments were performed to test for the interaction of these compounds with the nucleoprotein fraction. All results were negative. Cultures containing nucleoprotein fraction plus glutathione or cystine did not differ in size or microscopic appearance from those containing nucleoprotein alone. No information is yet available to indicate which factors in Mixture No. 199 are involved in

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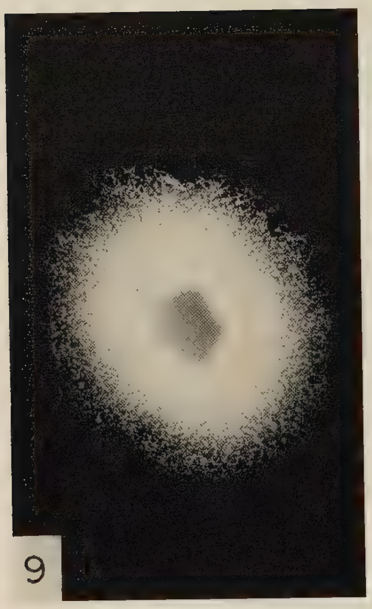
Figs. 5–8. Cellular detail after 5 days at margin of experimental cultures. × 112.
Fig. 5. Unsupplemented basal medium.
Fig. 6. Basal medium supplemented with nucleoprotein fraction only.
Fig. 7. Basal medium supplemented with barium-soluble dialysate only.
Fig. 8. Basal medium supplemented with both nucleoprotein and barium-soluble dialysate fractions.



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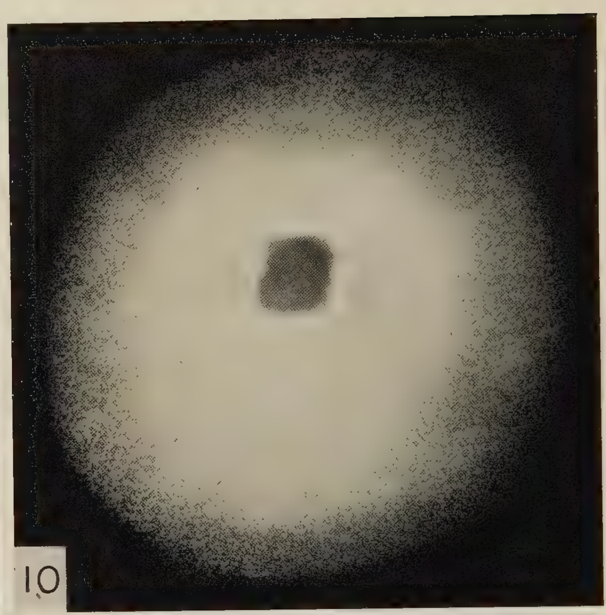

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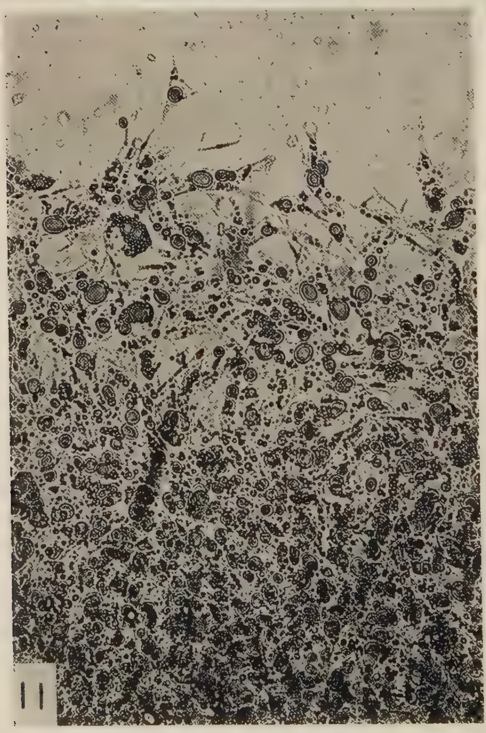

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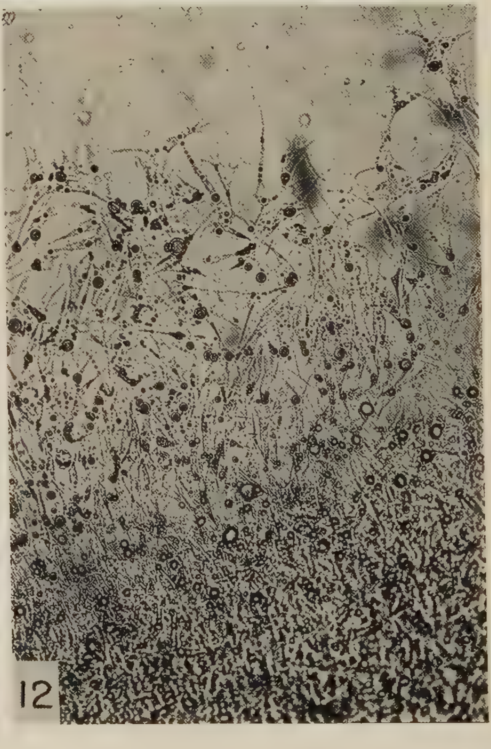

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TABLE II

Effects of nucleoprotein fraction and solution No. 199 on chick heart fibroblasts

Supplement	Amount added	Lactic acid[2] in supernatants at 7 days, micrograms/ml	Areal increase[3] at 10 days, mm²
None (basal medium[1])	—	203	10.0 ± 1.3
Solution No. 199	25%	387	28.6 ± 1.6
Nucleoprotein fraction	1.4 mg/ml	350	48.8 ± 6.9
Solution No. 199 plus nucleoprotein fraction	each as above	930	101.4 ± 9.7

the interaction with nucleoprotein, but elimination experiments should provide this data. It would also be of interest to study the synergism of nucleoprotein fraction in the absence of other proteins, e.g. — utilizing cell suspensions (5) as an assay system.

SUMMARY

1. Nucleoprotein and barium-soluble dialysate fractions have been prepared from chick embryo extract, using methods previously described. These fractions were assayed singly and in combination for growth-promoting activity, using cultures of chick heart fibroblasts and a dialyzed plasma-serum medium.

2. Cultures containing both fractions exhibited a sustained, even outgrowth, high population density in the marginal zone and normal cell morphology. In appearance these preparations closely resembled cultures containing fresh whole embryo extract.

3. The effects described could not be elicited with either nucleo-protein or dialysate fractions separately. Cultures containing nucleoprotein fraction

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[1] *Basal medium:* dialyzed chicken plasma + supernatant 35 per cent dialyzed horse serum, 65 per cent Gey's solution.

[2] Average values for duplicate determinations on pooled supernatants from six cultures.

[3] Mean value and standard error (21).

Figs. 9–10. Outgrowth at 10 days in experimental cultures. × 5.7.
Fig. 9. Basal medium supplemented with Mixture No. 199 only.
Fig. 10. Basal medium + nucleoprotein fraction and Mixture No. 199.
Figs. 11–12. Cellular detail after 5 days at margin of experimental cultures. × 117.
Fig. 11. Basal medium supplemented with Mixture No. 199 only.
Fig. 12. Basal medium + nucleoprotein fraction and Medium No. 199.

only showed moderate increases in surface area but the outgrowths became refractile and heavily vacuolated. Addition of the dialysate fraction alone gave thin spreading outgrowths with normal cell morphology but low population density.

4. Similar interaction with the nucleoprotein fraction was also demonstrated for a synthetic nutrient (Morgan-Parker Mixture No. 199). Cultures containing Mixture 199 alone showed small increases in surface area but were deficient in cell morphology.

We are indebted to Miss Carolyn Brode, Mr. Don Farquhar, Miss Miriam Lieberman, and Mr. Lawrence Resseguie for technical assistance in culture operations. Thanks are due also to Dr. Daniel Mazia for the use of a Spinco Ultracentrifuge in preparing test fractions.

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ANTIBIOTICS IN TISSUE CULTURE

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With the widespread use of antibiotics, it is becoming increasingly important to know the effects of these agents at the levels of the individual cells and tissues. This information is of interest not only to the clinician and the experimental worker attempting to establish therapeutic blood levels without damaging tissue, but to workers in any branch of research where the cytotoxicity of substances influences the controlled experiment and the interpretation of a given set of data.

To those working in tissue culture, the antibiotics offer a method of maintaining sterility in cultures. The ideal substance would necessarily have to meet very rigid requirements. Among these, the following are most important: a) It must not be toxic to living tissue in concentrations which would be adequate to inhibit bacterial contamination. b) It must not delay growth or alter the cells to any appreciable degree. c) It must be easily incorporated into the culture media.

METHODS AND MATERIALS

Aureomycin and terramycin studies were conducted using nine day chick embryo heart by the method described by Fusillo *et al.* (17), with the antibiotic incorporated directly into the culture medium.

In a second series of experiments, mouse heart was grown in antibiotic-free nutrient and exposed to aureomycin, terramycin, actidione or polymyxin in Gey's solution. Damage, as indicated by granulation and rounding of the cells, was read at 24 hours and in some instances at later periods. Growth was not measured by this method. Residual vitality was tested by removing the antibiotic and returning the tissue to an antibiotic-free nutrient.

RESULTS

The effect of terramycin on normal chick embryonic tissue is outlined in Table I. Growth was decreased with increasing concentrations. A concentration of terramycin in the nutrient medium, 3000 mcg/ml and greater, completely inhibited the growth of embryonic cells.

While growth of chick heart appeared to be diminished when the tissue

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TABLE I

Effect of terramycin on normal embryo heart tissue in tissue culture.

Concentration of terramycin mcg/ml		Average index of growth	Number of explants
Control	0	69.8 ± 1.4	41
	30	63.3 ± 3.6	15
	240	41.3 ± 2.4	16
	900	15.2 ± 0.48	18
	3 000	no growth	19

TABLE II

Effect of antibiotics on embryonic mouse heart tissue bathed in Gey's solution containing the respective antibiotics.

Antibiotic	Concentration of antibiotic initiating depression of growth mcg/ml	Concentration of antibiotic causing complete inhibition of growth mcg/ml	Remarks
Aureomycin . . .	80 mcg/ml	—	160 mcg/ml produced severe cytological damage
Actidione	8–10 mcg/ml	16-20 mcg/ml	cultures from 10 mcg/ml survived subculture
Polymyxin B . . .	250 mcg/ml	750 mcg/ml	cultures from 250 mcg/ml survived subculture
Terramycin . . .	100 mcg/ml	>200 mcg/ml	mouse heart exposed to to 150 mcg/ml did not affect pulsation

was placed in a nutrient containing increasing amounts of aureomycin HCl, measurements were difficult to evaluate because of a heavy precipitate which collected on the plasma clot. The latter precipitation was also observed by Lepine (26), but was not recorded by Antikajian (2) in her studies.

Mouse heart grown in antibiotic-free nutrient (Table II) for 24 hours and exposed to terramycin in Gey's solution in a concentration of 100 mcg/ml was perceptibly damaged. (Mouse heart grown in nutrient containing the antibiotic was unaffected at 100 mcg/ml.) At 150 mcg/ml in Gey's solution, some of the cell outgrowth was killed, but pulsation was not affected. Even at 48 hours two hundred mcg/ml produced only slight visible changes in the cells, but no cultures survived 48 hour exposure. Higher concentrations produced more severe visible damage.

Aureomycin HCl (Lederle, sodium glycinate buffered) at 80 mcg/ml in Gey's solution slightly damaged mouse heart tissue in 24 hours. Severe cytological damage was produced by 160 mcg/ml.

Studies with actidione in Gey's solution revealed slight damage to mouse heart at concentrations of 8 to 10 mcg/ml from which cultures subsequently recovered. At concentrations of 16 to 20 mcg/ml the 24 hour response was severe and no cells survived.

Polymyxin B in Gey's solution at 100 mcg/ml produced in 96 hours only a slight effect on mouse heart. A concentration of 250 mcg/ml produced marked cell damage in 24 hours. The cultures recovered readily from the latter dose. However, 24 hour exposure to 500 mcg/ml produced moderate damage and allowed only partial recovery of the cells. At 750 and 1000mcg/ml visible changes were severe and cultures did not recover.

DISCUSSION

The physiological effects of antibiotics in tissue culture are not clearly understood. While much experimentation is lacking, it remains that the effects of these drugs on living tissue, whether *in vivo* or *in vitro,* can have practical application.

Tables III and IV summarize some of the work that has been accomplished on the effects of antibiotics in tissue culture. It can be seen that the early data on the inhibitory effect of crude penicillin (10) was later clarified with advent of pure crystalline compounds. Actidione and patulin appear to be

TABLE III

Effect of antibiotics on chick embryo heart tissue in tissue culture.

Antibiotic	Concentration of antibiotic initiating depression of growth mcg/ml	Concentration of antibiotic causing complete inhibition of growth mcg/ml	Remarks
Streptomycin . . .	60 mcg/ml	90 000 mcg/ml	
Chloromycetin . .	480 mcg/ml	not completely inhibited at 1 200 mcg/ml	growth apppeared to be greater than controls at 240 mcg/ml
Polymyxin	100 mcg/ml	2 500 mcg/ml	
Viomycin	150 mcg/ml	30 000 mcg/ml	
Terramycin	30 mcg/ml	3 000 mcg/ml	relatively toxic
Aureomycin . . .	Growth appeared to be decreased but a heavy precipitate prevented accurate measurement.		

TABLE IV

Effect of antibiotics on various tissue in tissue culture.

Investigator	Antibiotic	Tissue	Concentration of antibiotic initiating depression of growth	Concentration of antibiotic causing complete inhibition of growth	Remarks
Fleming (16) 1929 . .	Penicillin	human leucocytes	none with 1 : 100 crude filtrate	—	—
Chain (9) 1940 . . .	»	»	tolerated 1 : 1 000 crude filtrate	—	—
Abraham (1) 1943 . .	»	»			1 : 250 was below the LD 50 for these cells
Jacoby (1) 1943 . . .	»	hen macrophages	1 : 500 with crude filtrate		recovery at 1 : 200
Medewar (1) 1943 . .	Penicillin purified	Chick ventricle, intest, kidney	arrested at 1 : 800	—	slight growth at 1 : 1 600
Gey (18, 19) 1945 . .	Penicillin	Rat and human normal and malignant	none with penicillium filtrates diluted 1 : 10		1 : 2 000 dilution cultures grew well
Herrell (21) 1943 . .	»	Rabbit lymphocytes	3–4 000 μ/ml	—	6–8 000 μ/ml reduced migration
Bucher (4, 5, 6) 1946	»	Rabbit fibroblasts	1 000 μ/ml	—	—
» 1946	»	»	625 μ/ml	—	tissue bathed in antibiotic solution
» 1947	Penicillin Lilly	»	100 μ/ml	—	produced metaphase block
» 1947	» Roche	»	200 μ/ml	—	produced metaphase block
» 1947	» pure	»	10–20 000 μ/ml	—	high conc. decrased mitosis
Cruickshank (12) 1952	Penicillin G	human skin	—	6 000 mcg/ml	normal cultures at 600 mcg/ml

Ikegaki (22)	»	chick heart	1 000 mcg/ml	100 000 mcg/ml	10–100 mcg/ml grew more than controls
Cruickshank (12) . .	»	human skin	—	10 000 mcg/ml	normal cultures obtained at 1 000 mcg/ml
Lepine (26) 1950 . .	Aureomycin	chick heart	1 mcg/ml	1 000 mcg/ml	
Antikajian (2) 1951 .	»	»	0.1 mcg/ml		
Keilova-R (31) 1951	»	chick heart, aorta, frontal bone	500 mcg/ml	greater than 4 000 mcg/ml	much cellular damage at 4 000 mcg/ml
Cruickshank (12) 1952	»	human skin	greater than 200 mcg/ml	—	at 1 mcg/ml a ppt was observed
»	Polymyxin E	»		10 000 mcg/ml	normal cultures obtained at 2 000 mcg/ml and below
»	Polymyxin	»		1 000 mcg/ml	normal growth at 200 mcg/ml
Vollemar (37) 1947 .	Patulin	chick heart	1 : 10 000 dilution	1 : 5 000 dilution	1 : 25 000 to 1 : 50 000 dilution stimulated growth
»	»	mouse leucocytes		1 : 5 000	more rapid migration at 1 : 25 000 to 1 : 50 000
Keilova (23) 1949 . .	»	chick heart	2×10^{-6} mcg/ml	—	—
Lepine (26) 1950 . .	Chloromycetin	»	10 mcg/ml	1 000 mcg/ml	
Keilova-R (31) 1951 .	»	»	100 mcg/ml	1 000 mcg/ml	
Cruickshank (12) 1952	»	human skin	2 mcg/ml	100 mcg/ml yielded abnormal cultures	greater than 10 mcg/ml are likely to prove toxic
»	Terramycin	»	200 mcg/ml	1 000 mcg/ml	with 20 mcg/ml normal cultures were obtained
»	Neomycin	»		10 000 mcg/ml	normal cultures at 1 000 mcg/ml

quite toxic for cells in tissue culture. Both of these drugs are correspondingly toxic to man. For streptomycin, results seem to indicate a pharmacologically active effect of enhancement of growth at concentrations less than 100 mcg/ml. It must be remembered that the selection of resistant bacterial variants to streptomycin is a "one step" type of resistance with the resulting organism several thousand fold more resistant than its parent.

From the data presented, aureomycin and terramycin would not appear to be applicable to the needs of the tissue culture worker. However, from studies with chloromycetin on chick embryo tissue, this substance appears to have little toxicity. The development of antibiotic resistant variants of bacteria to chloromycetin is a slow stepwise increase and concentrations which do not appear toxic to the host cell would usually kill most bacterial contaminants.

This problem of maintaining bacteriologically sterile tissue cultures has been one of great concern among tissue culture workers. Attempts at growing excised fragments of tumor or even embryonic normal tissue have many times proved futile because of bacterial contamination. Recently, Enders (14) in his poliomyelitis virus studies successfully used antibiotics as a method of preventing contamination in bovine amniotic fluid.

Another consideration is that antibiotics may serve a dual purpose in a cell-infectious agent relationship. While we have always considered the infectious agent – antibiotic relationship, we have not usually considered the host cell – antibiotic relationship. The observations of Stowens (35) and Schwachman (34), in their studies of fibrocystic disease of the pancreas certainly exhibit a host cell – antibiotic relationship in which a clinical response was produced in a disease which is considered non-infectious. Carvalho (7) in preliminary manometric studies on the effect on respiration of chick embryo tissue found maximum growth and maximum respiration at a dilution of 10-1 of Merck streptomycin. It is true that the respiratory quotient does not in all cases reflect increased or decreased growth, but certainly here is a basis for more advanced tissue metabolism studies.

In the treatment of skin diseases, irritants have always been a problem. The antibiotics offer an invaluable aid to the armamentarium of the dermatologist. But, if the antibiotic is more cytotoxic than antibacterial, the purpose is defeated.

Dubin (13) has recently grown malaria parasites in chick macrophage tissue cultures. In these studies the problem of bacterial contamination seemed to be his major problem. Despite extensive studies with penicillin and streptomycin in various concentrations, one fails to discern whether or not this contaminant was constant — and whether or not the contaminating organism was natively resistant to the antibiotics used. These studies revealed that high concentrations of streptomycin were toxic to macrophages. These latter difficulties may have been overcome by using another antibiotic or combination other than penicillin and streptomycin. In our experience with surgical and autopsy material, chloromycetin (240 mcg/ml) has proved very valuable in maintaining sterility. In some tumors where the contamination ensued, the contaminant was found to be a pseudomonad. Polymyxin would appear to be the antibiotic of choice for such material.

Hitherto, we have always measured the *in vitro* effect of antibiotic on a particular microorganism without regard to the phagocytic mechanism. Studies by Baker (3), Rogers and Pompsett (32) demonstrated the survival of staphylococci within living macrophages. Mackaness (28) using *M. tuberculosis* infected macrophage suspensions tested the effect of various drugs and found that the intracellular bacteria required about 10 to 75 times as much drug to kill as in the usual culture medium. With respect to terramycin, the organism required 12.5 mcg/ml intracellularly as well as extracellularly. However, terramycin was the most toxic agent to the host cell. Studies with isoniazid (29) also revealed that isoniazid was equally active against intracellular bacilli and those grown in the bacteriological medium.

Suter (36) has described a method for infecting monocytes which have been transformed into macrophages with *M. tuberculosis.* He also found that higher concentrations of antibiotic were necessary to inhibit the bacteria within the cell than without. Virulent bacteria in these experiments caused more damage to the host cell than avirulent strains. In this type of *in vitro* experiment, the damage of the bacteria to the cell must be considered in the evaluation of the antibiotic toxicity.

The application of academic findings of the effects of antibiotics on tissue in *in vitro* tissue culture are more diversified than one anticipates. Only a few instances of the practical application have been cited.

SUMMARY

Growth of chick fibroblasts was completely inhibited by 3000 mcg of terramycin per millimeter of nutrient media.

Aureomycin in Gey's solution, when added to growing cultures of mouse fibroblasts, caused marked cytologic changes at concentration of 160 mcg/ml.

Actidione in a concentration of 16 to 20 mcg in Gey's solution, when added to growing mouse fibroblasts, produced severe cytologic changes.

Terramycin, aureomycin, and polymyxin in Gey's solution were more toxic (when added directly to growing cells) than when incorporated into the nutrient medium.

Extraordinarily high concentrations of antibiotics have been shown to inhibit the proliferation of fibroblasts and inflict tissue damage to cells in (tissue) cultures.

Certain antibiotics, or specific combinations of antibiotics, have proved to be satisfactory in maintaining sterility of tissue cultures (33, 14).

The major antibiotics that are widely used today are not cytotoxic in concentrations much higher than those usually attainable in body fluids.

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STUDIES OF NUCLEIC ACID METABOLISM IN EMBRYONIC TISSUE CULTURE WITH THE AID OF C^{14}-LABELED PURINES[1]

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Publications from this laboratory have emphasized the use of tissue culture methods for biochemical investigations of cells under controlled conditions (5, 9). Up to the present time isotopic studies concerned with the biosynthesis and intermediate metabolism of nucleic acids have employed only radiophosphorus, P^{32} (7). The important advances resulting from the use of purines and pyrimidines labeled with radiocarbon, C^{14}, in animal experiments suggest the value of these tools for *in vitro* systems. Thus, it has been shown that, following the administration of isotopic adenine to rats, both adenine and guanine of the tissue nucleic acids become labeled; but isotopic guanine is not effectively utilized for nucleic acid synthesis in several species (2). The indications are that guanine is most effective at the nucleotide level, i.e., as guanylic acid, derived from adenine via adenylic acid. In many studies emphasis is placed on the relative rates of renewal of ribose- and desoxyribose-nucleic acids (RNA and DNA) in various tissues. In this connection, the possible interconvertability of these two substances is receiving attention.

The present paper is concerned with the relative rates of incorporation of C^{14}-labeled adenine and guanine into DNA and RNA of chick heart fibroblasts. In studying the pathway of nucleic acid synthesis, nutrient media have been employed with both free- and polynucleotide-bound labeled purines. For the latter type of experiment, isotopic adenine was injected into fertile eggs, and radioactive DNA and RNA preparations subsequently isolated. Each was shown to be labeled in both its purine constituents. By employing these nucleic acids in the embryonic extract (EE) medium, it has been possible to study the transfer of bound adenine and guanine from the nutrient solution to the explanted tissue.

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MATERIALS AND METHODS

Adenine-8-C^{14} and guanine-8-C^{14} with specific activities of 1.0 and 2.2 mC per mM, respectively, were purchased from the Isotopes Specialties Co., Glendale, California, on allocation from the US. Atomic Energy Commission. Their radiochemical purity was at least 95 per cent, as determined by chromatography on starch columns.

Labeled nucleic acids were prepared as follows: 3.9 μc of adenine-8-C^{14} (0.6 mg) were injected into each of a number of 9-day fertile eggs. Four days later the embryos were dissected, and used for the isolation of DNA and RNA by the method of Hurlbert and Potter (8). The final preparations were free of protein, as judged by negative ninhydrin tests, and each displayed its characteristic ultraviolet absorption spectrum. The DNA gave a negative orcinol test (for RNA), and the RNA a negative diphenylamine reaction (for DNA).

Neutral solutions of the adenine-8-C^{14}, guanine-8-C^{14}, labeled DNA[1], and labeled RNA were sterilized by autoclaving, prior to addition to the EE medium. The latter represented a centrifuged 25 per cent extract of whole 13-day chick embryos (5). Embryonic chick heart fragments were cultivated in this nutrient according to the quantitative roller tube method previously described (5), with changes of medium at 2-day intervals. A cultivation period of 6 days was employed, since this interval represented the time of maximum cellular proliferation (7).

Approximately 20 tubes were used in each tissue culture experiment, in order to provide sufficient nucleoprotein for isolation purposes. After removal of the tubes from the roller, the medium was aspirated off, and the cultures washed with saline. The tissues were pooled, and a radioactive nucleoprotein fraction isolated (8). A portion of the latter was then converted to sodium nucleates. The specific radioactivities of both fractions were determined by counting weighed solid layers (5). The observed counts per minute per mg were converted to corresponding μM of labeled adenine or guanine, with the aid of the known specific activities of these purines.

A carrier method was then employed for the isolation of pure nucleic acids from the main nucleoprotein fraction. To the latter was added 4 to 8 parts of a stock non-labeled nucleoprotein preparation (from 13-day embryonic thick tissues). The resulting isotopically-diluted material (about 0.5 to 1 gm in quantity) was used to isolate DNA and RNA. The radioactivities of these nucleic acids were determined by counting aliquots of solutions dried on glass cover slips. The appropriate numerical factor was used to convert these values into the original activities of the DNA and RNA of the heart cultures. As before, the results were expressed in μM of labeled purine incorporated, per mg nucleic acid.

In order to determine the C^{14} distribution in the isolated nucleic acids, 3 to 4 mg of the latter were hydrolyzed in 1 N HCl for 1 hour at 100°. The hydrolysates were subjected to starch column chromatography, according to the Daly-Mirsky modification (3) of the Moore-Stein procedure. One ml fractions of effluent were collected in the purine regions, and were individually transferred to glass cover slips, dried, and counted. Subsequently each cover slip was rinsed with 0.1 N HCl, and the dissolved purine measured in the Beckman spectrophotometer at appropriate wave length.

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[1] The DNA solution could not be filtered, because of its collodial character. There was no evidence of decomposition upon autoclaving, as judged by the complete reprecipitability of the DNA upon addition of acid.

In measuring radioactivity, the stronger samples were determined in the mica window Geiger counter, while with samples of relatively weak radioactivity, the windowless gas-flow counter was used, and sufficiently long counting times were employed to reduce the probable error to 2 to 5 per cent.

RESULTS AND DISCUSSION

The addition of labeled adenine to EE medium resulted in a rather extensive incorporation of C^{14} into the nucleoprotein of heart cultures (Table I). The value, 15.4 μM represents a utilization by the tissue of about 15 per cent of the total radioactivity employed in the three changes of nutrient. By contrast, the 0.7 μM value in the labeled guanine experiment corresponds to an uptake of less than one per cent of the total C^{14}. This marked difference between the degrees of utilization of free adenine and guanine for nucleic acid synthesis in tissue culture is in agreement with *in vivo* findings for several animal species (2).

Following removal of the protein component, the mixed nucleic acid fraction from the adenine-8-C^{14} experiment exhibited enhanced activity. Upon resolution of this fraction to its components, the specific activity of the DNA was 2.2 times that of the RNA. However, chemical analysis indicated 65 μgm of RNA, but only 30 μgm of DNA, per mg nucleoprotein. Accordingly the C^{14} was incorporated approximately equally into the two types of nucleic acids. From the 93 μM value it may be calculated that approximately 7 per cent of the total purines in the DNA were labeled, while the corresponding RNA value (42 μM) reflects a 3 per cent labeling.

TABLE I

Relative rates of incorporation of free labeled purines into nucleic acids of heart cultures.

Adenine and guanine were added to the 25 per cent EE at 0.100 and 0.109 mM concentrations, respectively.

Purine added to medium	μM incorporated per gm isolated material				
	Nucleo-protein	Protein	Mixed sodium nucleates	DNA	RNA
Adenine-8-C^{14}	15.4	< 0.1	68	93	42
Guanine-8-C^{14}	0.7			1.3	1.9

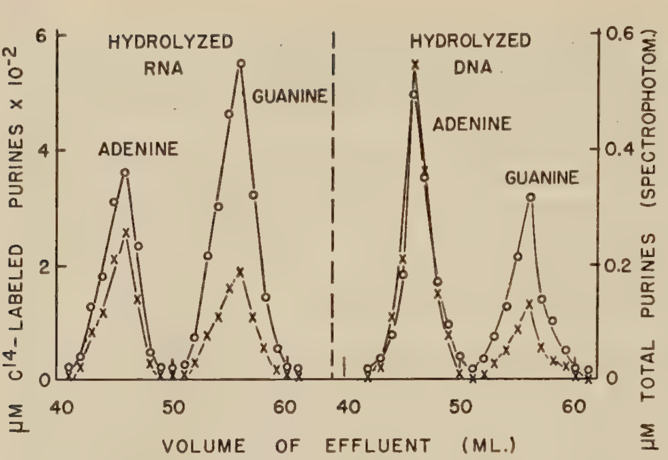

Fig. 1. Chromatograms of nucleic acids isolated from embryonic chick heart cultivated in the presence of adenine-8-C^{14}. O-spectrophotometric determinations; X-radioactivity.

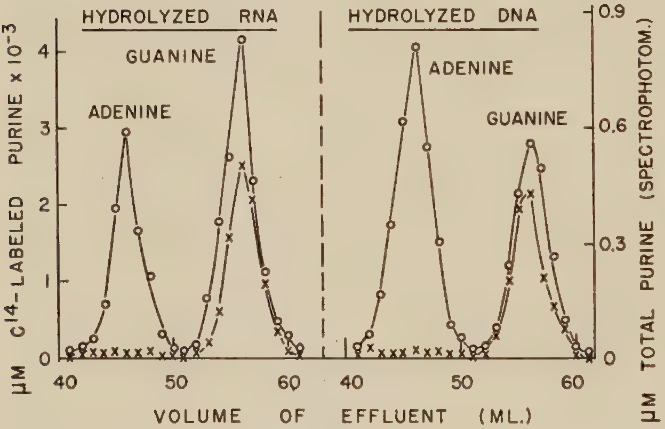

Fig. 2. Chromatograms of nucleic acids from heart cultivated in the presence of guanine-8-C^{14}. O-spectrophotometric determinations; X-radioactivity.

In the guanine-8-C^{14} experiment, both DNA and RNA had very low radioactivity. However, RNA was higher in both specific activity and total C^{14} content (per mg nucleoprotein) than DNA.

Figs. 1 and 2 indicate the total purine concentrations and the distribution of C^{14} between adenine and guanine in the RNA and DNA preparations of Table I. In no case was there significant radioactivity in the pyrimidine regions (not shown) of the chromatograms.

The results are interpreted in Table II. Considering first the adenine-8-C^{14} experiment, it may be seen that both the adenine and guanine had higher C^{14} concentration in DNA than in RNA. The values 0.053 and 0.0325 indicate that approximately 5 per cent of the adenine and 3 per cent of the guanine residues in the RNA were labeled. The corresponding percentages were about 9 and 4 for DNA. These figures agree reasonably well with the average labeled purine values calculated for RNA and DNA from Table I. It will be indicated later that the polynucleotides of the EE were actually the major source of purines for tissue nucleic acid synthesis in the fibroblasts, particularly DNA.

The higher activities in DNA appear to be characteristic of growth processes. Thus regenerating liver and tumor tissue incorporate adenine rapidly into DNA *in vivo*, while in non-growing organs, such as liver, RNA has a higher turnover rate (1).

In the last column of the upper half of Table II the adenine to guanine C^{14} ratios differed markedly for RNA and DNA. This difference may be due to the existence of discrete metabolic pools of nucleotides for RNA and DNA synthesis.

The presence of guanine-8-C^{14} in the nutrient medium (lower half of Table II) led to low C^{14} concentrations in the guanine of both RNA and DNA, and almost negligible radioactivities in adenine.

Experiments such as those of Friedkin (6) with isotopic thymidine have indicated the usefulness of the embryonated egg for the biosynthetic preparation of labeled nucleic acids in relatively large quantities. Such preparations were needed in the present study for determinations of the utilization of polynucleotide purines in tissue culture media. Following the injection of adenine-8-C^{14} into fertile eggs (Table III), approximately 14 per cent of the radioactivity was recovered in 2 gm of nucleoprotein, isolated from the embryos. By way of comparison, thymidine-C^{14} (but not thymine) was incorporated into DNA of chick embryos to the extent of 23 per cent, between the fifth and eighth days of incubation (6).

As in the cultivation experiments of Table I, the DNA in Table III had

TABLE II

Summary of chromatographic data of Figs. 1 and 2.

Purine added to medium	Nucleic acid isolated	Ratio of labeled to total purine		Ratio of adenine-C^{14} to guanine-C^{14}
		Adenine	Guanine	
Adenine-8-C^{14}	RNA	0.053	0.0325	0.96
	DNA	0.095	0.0415	3.20
Guanine-8-C^{14}	RNA	0.0004	0.0032	0.08
	DNA	0.0001	0.0040	0.07

TABLE III

Incorporation of labeled adenine into nucleic acids of chick embryos.

Six 9-day embryonated eggs were each injected with adenine-8-C^{14}, and 4 days later the embryos were harvested.

μM C^{14}-purines per gm isolated nucleic acid fractions[1]			
Nucleoprotein	Mixed sodium nucleate	DNA	RNA
1.5	5.4	7.8	3.2

Chromatographic analysis of nucleic acids			
Nucleic acid	Ratio of labeled to total purine		Ratio of adenine-C^{14} to guanine-C^{14}
	Adenine	Guanine	
RNA	0.0050	0.0042	0.75
DNA	0.0070	0.0060	1.17

a higher specific activity than RNA. The chromatographic analyses indicated that from 0.4 to 0.7 per cent of each purine was labeled. The adenine to guanine C^{14} ratios differed somewhat from those in the tissue culture experiments, as might be expected.

Because of extensive dilution by nucleic acids in the eggs, the radioactivities of the isolated DNA and RNA in Table III were relatively low. However, the C^{14} concentrations were adequate for cultivation experiments, as is indicated in Table IV.

In the second column of this Table, the value, 16, represents an uptake into tissue nucleoprotein of 11 per cent of the total RNA-C^{14} employed in the medium, while 40 corresponds to a 13 per cent utilization of the DNA-C^{14}. These figures are comparable in magnitude to the 15 per cent uptake of free adenine-8-C^{14}, mentioned in connection with Table I.

It is significant that both DNA and RNA of the cultures became radioactive when either type of labeled nucleic acid was present in the EE. The most probable assumption is that at least partial hydrolysis occurred, either in the medium, or following the absorption of the original labeled nucleic acid into the tissue cells.

With either labeled RNA or DNA in the nutrient, the resulting DNA to RNA C^{14} ratio of the cultures was greater than unity, but in view of the

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[1] No carrier was required in these isolations.

TABLE IV

Relative rates of incorporation of C^{14}-labeled DNA and RNA into nucleic acids of heart cultures.

The radioactive DNA and RNA preparations of Table III were employed. The theoretical specific activities of adenine and guanine were 915 and 785 counts/min./μM for the DNA, and 655 and 550 cm/μM for the RNA (calculated from the chromatographic data of Table III). The averages of these pairs of values, 850 and 600 were used to calculate μM of purines in each fraction.

Labeled nucleic acid added per 100 ml of 25 per cent EE	μM of labeled purines incorporated per gm isolated material			Ratio of DNA-C^{14} to RNA-C^{14}	Chromatographic ratio of adenine-C^{14} to guanine-C^{14}
	Nucleoprotein	DNA	RNA		
20 mg RNA	16	81	50	1.6	0.63 (for RNA)
25 mg DNA	40	412	176	2.3	3.25 (for DNA)

greater abundance of RNA in the cultures (previously mentioned), it may be concluded that the RNA utilized as much of the labeled nucleic acid of the medium as did DNA, in tissue synthesis.

Calculated in terms of percentage labeling, the nucleic acid values indicate that, with radioactive RNA in the medium, 6 and 3.7 per cent of the purines in DNA and RNA, respectively, became labeled. When radioactive DNA was employed, the corresponding percentages were 30 and 13. These relatively high values cannot safely be interpreted as indicating corresponding degrees of net nucleic acid synthesis, since probably turnover processes were also operative.

It is of interest that the adenine-to-guanine C^{14} ratio of the tissue RNA (0.63) differed only slightly from that of the original labeled RNA of the medium, 0.75 (Table III), while the corresponding ratios for the DNA were 3.25 and 1.17 (Table III). In considering these changes it is likely that hydrolysis of the labeled nucleic acids released isotopic adenine and guanine nucleotides. The isotopic adenylic acid could then form additional guanylic acid, and this process would favor a decrease in the adenine-to-guanine C^{14} ratio of the tissue nucleic acids. However, the lowering observed for RNA was insignificant, while the ratio *increased* with DNA.

The interpretation of the present results is rendered complex by several factors:

1) The exact degrees of hydrolysis of the labeled nucleic acids are not known. Probably free purines were not released. If this had occurred, guanine could not be re-utilized effectively.

2) The bound labeled purines in the medium were utilized for *both* DNA and RNA synthesis. Probably adenine was incorporated in preference to guanine into tissues, as in Table III.

3) The nucleic acids normally present in the EE are known to play an important role in the growth of the cultures. As the studies of Davidson and co-workers (4) indicate, the DNA and (particularly) the RNA content increases in heart fibroblasts cultivated in embryonic extract medium.

4) Recent chemical studies suggest that both DNA and RNA are heterogeneous substances of exceedingly complex structure.

Studies now in progress in this laboratory are concerned with the further elucidation of the pathway of nucleic acid synthesis in tissue culture, with emphasis on the exact nutritional requirements of embryonic cells, and the effect of experimental variations in the environment on synthetic processes.

SUMMARY

C^{14}-labeled adenine was readily utilized for nucleic acid synthesis in embryonic chick heart cultures. DNA and RNA, isolated from these cultures, contained radioactivity in both adenine and guanine constituents. C^{14}-labeled guanine was relatively poorly utilized, and was not significantly converted to polynucleotide adenine.

Labeled DNA and RNA, prepared by injecting C^{14}-adenine into fertile eggs, were used to study the transfer of bound purines from the nutrient medium to the cultivated tissue. The DNA, containing isotopic adenine and guanine, gave rise to double labeling in both the DNA and RNA of the cultures. Similar results were obtained with radioactive RNA in the medium. These findings were interpreted as indicating hydrolysis of the nucleic acids, with a subsequent utilization of resulting nucleotides for tissue synthesis.

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Experimental Cell Research, **6**, *353–360 (1954)*

OBSERVATIONS ON THE STRUCTURE OF *LEISHMANIA DONOVANI* — THE KALA-AZAR PARASITE

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Leishmania donovani, the Kala-azar parasite, was studied with the electron microscope by Emmel, Jakob and Golz (5) and Das Gupta, Bhattacharya and Sen-Gupta (2). These studies, however, offered little information regarding internal details of the parasites as the protozoa are generally so thick that in direct electron micrographs they appear uniformly dark. Löfgren (7) tried to reduce the opacity by disrupting the flagellates by suspension in distilled water. This method suffered from the disadvantage that it produced a great distortion in the morphology.

In the present paper a method is described which renders the parasites suitable for electron microscopy but does not result in any distortion. With the help of this method it has been possible to observe many interesting features in the morphology of the leptomonad form of *Leishmania donovani*.

MATERIAL AND METHODS

The flagellate form of *Leishmania donovani* was produced by culture in N.N.N. (Novy, McNeal, Nicolle) medium of the spleen or bone marrow material obtained by puncture from Kala-azar cases. The flagellates growing in the water of condensation on the surface of the medium were used in this work.

Phase microscopy. Phase micrographs of the living parasites were made with Spencer dark contrast equipment. For this purpose, a drop of the water of condensation with the parasites was diluted with normal saline containing a little cocaine. A drop of this solution was put on a glass slide, a cover glass was superposed and pressed in order to produce as thin a film of suspension as possible. The flagellates were then continuously observed under the microscope and just when movement ceased micrographs were made.

Electron microscopy. The electron microscope used in the present investigation was one assembled at the Institute of Nuclear Physics, Calcutta (3). Fifty kilo-volt electrons were used throughout this investigation.

The specimens for electron microscopy were first prepared in the following way. The water of condensation containing the flagellates was diluted with citrated saline and a drop of this mixture transferred directly to specimen grids coated with collodion films. The specimens were fixed by exposure to the vapour of two per cent

OsO_4 solution and washed thoroughly in order to remove the salt crystals. This method had ultimately to be given up, as in the resulting electron micrographs, the flagellates appeared to be uniformly dark without any visible internal structure (2).

The method that succeeded in making the specimens sufficiently thin for electron microscopy without any alteration in the morphology, was the method of hydrolysis in normal HCl of specimens fixed in OsO_4. In this method the water of condensation with the flagellates was first smeared on collodion coated glass slides. The slides with the specimens were then fixed by exposure to OsO_4 vapour after which they were hydrolysed in normal HCl at 60° C for 30 minutes. After a thorough wash, the slides were allowed to dry and the collodion films with the parasites were stripped from the glass slide and mounted on specimen grids for introduction into the electron microscope. In some cases the specimens for electron microscopy were shadowed obliquely with gold-palladium vapour for enhancement of contrast.

Control experiments. In order to understand the effects of hydrolysis on the specimens, several control experiments were performed. In the first experiment, a smeared slide was hydrolysed in normal HCl as in electron microscopy. The density distributions in the hydrolysed specimens were then compared with those in normal unhydrolysed ones, under the dark contrast phase microscope. In another experiment, two slides with the smear of the flagellates were fixed simultaneously in OsO_4. One of the slides was hydrolysed in normal HCl at 60° C for thirty minutes, the other remaining as control. Both slides were then simultaneously stained in Giemsa and a careful comparison was made of the intensity of the stain in different regions of the body. The same experiment was also repeated with J.S.B. (10) stain.

OBSERVATIONS

Phase micrographs of the living specimens (Figs. 1, 2) show an oval shaped light nuclear zone (N) surrounded by comparatively dark cytoplasm. Within the light nucleus a dark body can often be seen which is the karyosome (K). The anterior tip of the body from where the flagellum emerges appears to be the darkest region in the whole cell. In dark phase micrographs the lighter regions correspond to regions of smaller optical path in the object. Hence, the lighter nuclear zone in the phase micrographs proves that the optical path corresponding to the nuclear site, is less than that of the cytoplasm.

Compared with the living specimens, the phase micrographs of fixed and hydrolysed controls (Fig. 3) show a reversal of contrast pattern. The nuclear site (N) is now definitely darker. This means that as a result of hydrolysis the cytoplasmic portions are more rapidly affected than the nuclear regions.

This fact is confirmed by a comparison of the intensity of Giemsa stain taken by hydrolysed and unhydrolysed specimens (Figs. 4, 5). The cytoplasm is stained deeply in the non-hydrolysed specimens (Fig. 4) while it takes only a very faint stain after hydrolysis (Fig. 5). A hydrolysed specimen stained with J.S.B. (Fig. 6) again shows faintly stained cytoplasm within which the nucleus (N) with karyosome (K) can be differentiated which is not possible in non-hydrolysed specimens similarly stained. These control experiments prove definitely that the main effect of hydrolysis in normal HCl at 60° C for thirty minutes is the preferential removal of the basophilia from the cells.

The *electron micrographs* of specimens fixed in OsO_4 vapour but not subjected to hydrolysis do not exhibit any internal structure (Fig. 7). The whole body is generally found to be opaque to the electron beam. Fig. 8 shows the great difference made by the process of hydrolysis described above. Electron micrographs of such fixed and hydrolysed specimens give clear representation of the internal details of the flagellate. The oval shaped nucleus (N) with a dense peripheral ring, a dark centrally situated karyosome (K) and the banana shaped parabasal body (P) slightly concave to the flagellar end are clearly visible. Just opposite the centre of the parabasal body and in line with the flagellum a dark body is visible which is the blepharoplast (B). The flagellum somewhat flattened as a result of hydrolysis is found to be more opaque to the electron beam at the point of its emergence from the body possibly due to its reinforcement by the periplast.

Longitudinal myoneme fibrils, arranged in bundles with branching and interconnection between them can be seen in more thoroughly hydrolysed specimens (Figs. 9 and 10). The interconnection between the myoneme fibrils can be seen more clearly when the flagellate is stretched in the transverse direction (Fig. 10).

The single flagellum usually noticed in normal specimens is found to resolve into a number of fine fibrils after hydrolysis; of these a maximum number of five fibrils may be counted in Fig. 9, eight in Fig. 10 and nine in Fig. 11. In the last figure, the flagellum is intact in its proximal part. It first resolves into two unequal bundles and two intermediate fibrils. One of the bundles separates into five fibrils of about 450 Å thick while the distal tip of the other bundle is found to fray into two more fibrils. The maximum number of fibrils counted is therefore nine. No distinct blepharoplast is visible; instead the flagellum seems to end inside the body in a rhizoid fashion (cf. Fig. 9).

A maximum number of nine distinct fibrils has been counted also in other cases (Figs. 13 and 14). In those cases of flagella-breakage where a smaller number of component fibrils have been observed (Figs. 9 and 10, and Fig. 12), some of the fibrils appear to be definitely thicker as if capable of further sub-division. The nine fibrils in Fig. 13 are arranged in a tuft and seem to be slightly twisted (cf. Figs. 9, 11). The proximal part of the flagellum is observed to remain intact in most of the cases while the fraying effect is remarkable at the distal tip often giving rise to a finger-tip appearance (Fig. 14). Some of the remnants of the protoplasmic binding material (C and D) may be seen to remain attached to different parts of the individual fibrils which cannot then be well resolved (Figs. 12, 14).

DISCUSSION

The phase micrographs of the living flagellates show that the optical path corresponding to the nuclear sites is less when compared to the rest of the cell, a fact which has also been observed with the rod shaped bacteria *E. coli* (4, 11).

A comparison of the phase micrographs of hydrolysed and non-hydrolysed specimens and the photomicrographs of similar specimens stained in Giemsa gives clear evidence of the selective removal of the cytoplasmic basophilia as a result of hydrolysis in normal HCl (12).

Figs. 1 and 2. Phase micrographs of living specimens of *Leishmania donovani*, N nucleus, K karyosome, V vacuole. Notice the oval shaped lighter nuclear zones surrounded by the denser cytoplasm. Magnification: 3500 ×.

Fig. 3. Phase micrograph of a specimen fixed in OsO_4 vapour and hydrolysed in normal HCl at 60° C for 30 minutes. The nucleus N is now darker than the surrounding cytoplasm. Magnification: 3500 ×.

Fig. 4. Photomicrograph of a specimen fixed in OsO_4 vapour and stained in Giemsa. Magnification: 3500 ×.

Fig. 5. Photomicrograph of a specimen fixed in OsO_4 vapour, hydrolysed in normal HCl at 60° C for thirty minutes and stained in Giemsa. Compared with the previous figure cytoplasm is found to be less deeply stained. Magnification: 3500 ×.

Fig. 6. Fhotomicrograph of specimen fixed in OsO_4 and hydrolysed in normal HCl at 60° C for thirty minutes, stained in J.S.B. Cytoplasm is found to be very faintly stained when compared with unhydrolysed stained specimens. Magnification: 3500 ×.

Fig. 7. Electron micrograph of *Leishmania donovani* fixed with OsO_4 vapour. Notice that the whole body is uniformly dark without any evidence of internal structure. Magnification: 8000 ×.

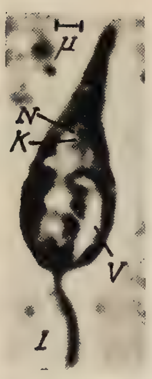
Fig. 8. Electron micrograph of specimen fixed in OsO_4 vapour and hydrolysed in normal HCl at 60° C for 30 minutes. Notice the improvement produced by the hydrolysis. The nucleus N, karyosome K, parabasal body P are now clearly visible. Magnification: 10,000 ×.

Figs. 9–14. Electron micrographs of *Leishmania donovani* flagellates fixed in OsO_4 and hydrolysed in normal HCl at 60° C for thirty minutes. All specimens shadowed with gold palladium.

Fig. 9. Bundles of longitudinal myoneme fibrils visible on the periplast and the flagellum splitted into five fibrils. Magnification: 7000 ×.

Fig. 10. A transversely stretched flagellate showing clearly the branching and interconnection between the myoneme fibrils. The flagellum in this case is resolved into 8 fibrils. Magnification 7000 ×.

Fig. 11. The body of the flagellate completely disorganised and the flagellum resolved into two bundles and two intermediate fibrils. Total number of fibrils counted is nine. Magnification 6000 ×.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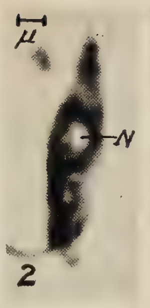
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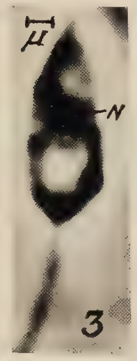
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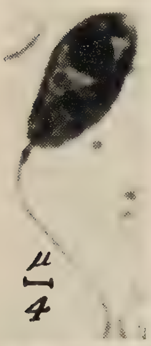
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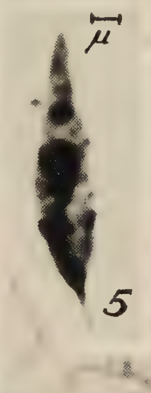
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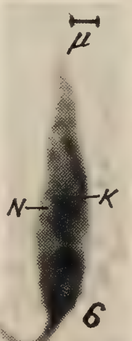
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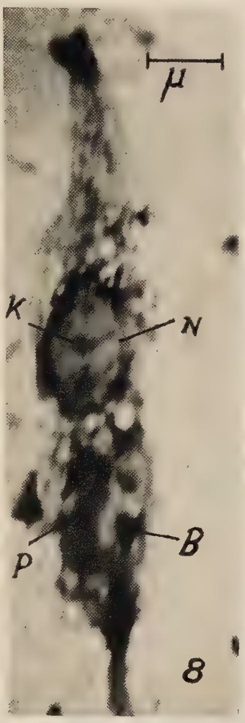
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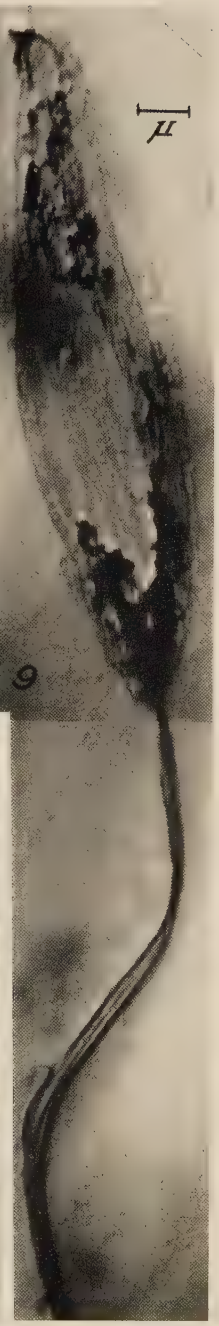
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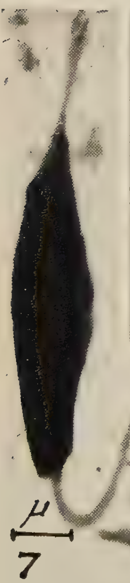
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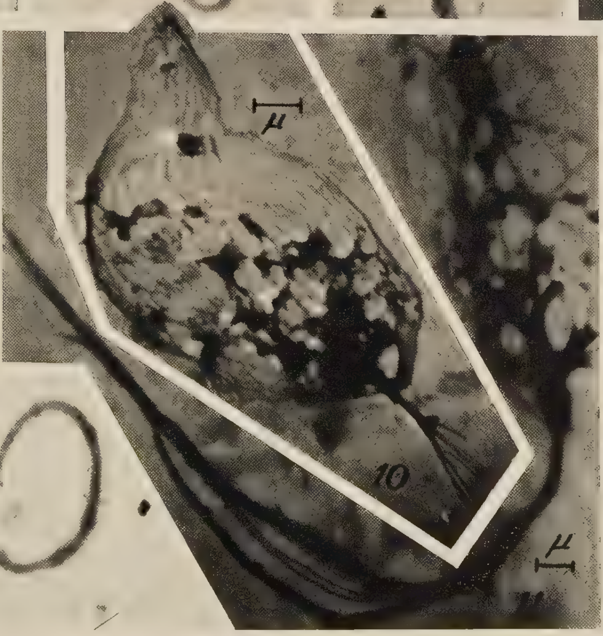
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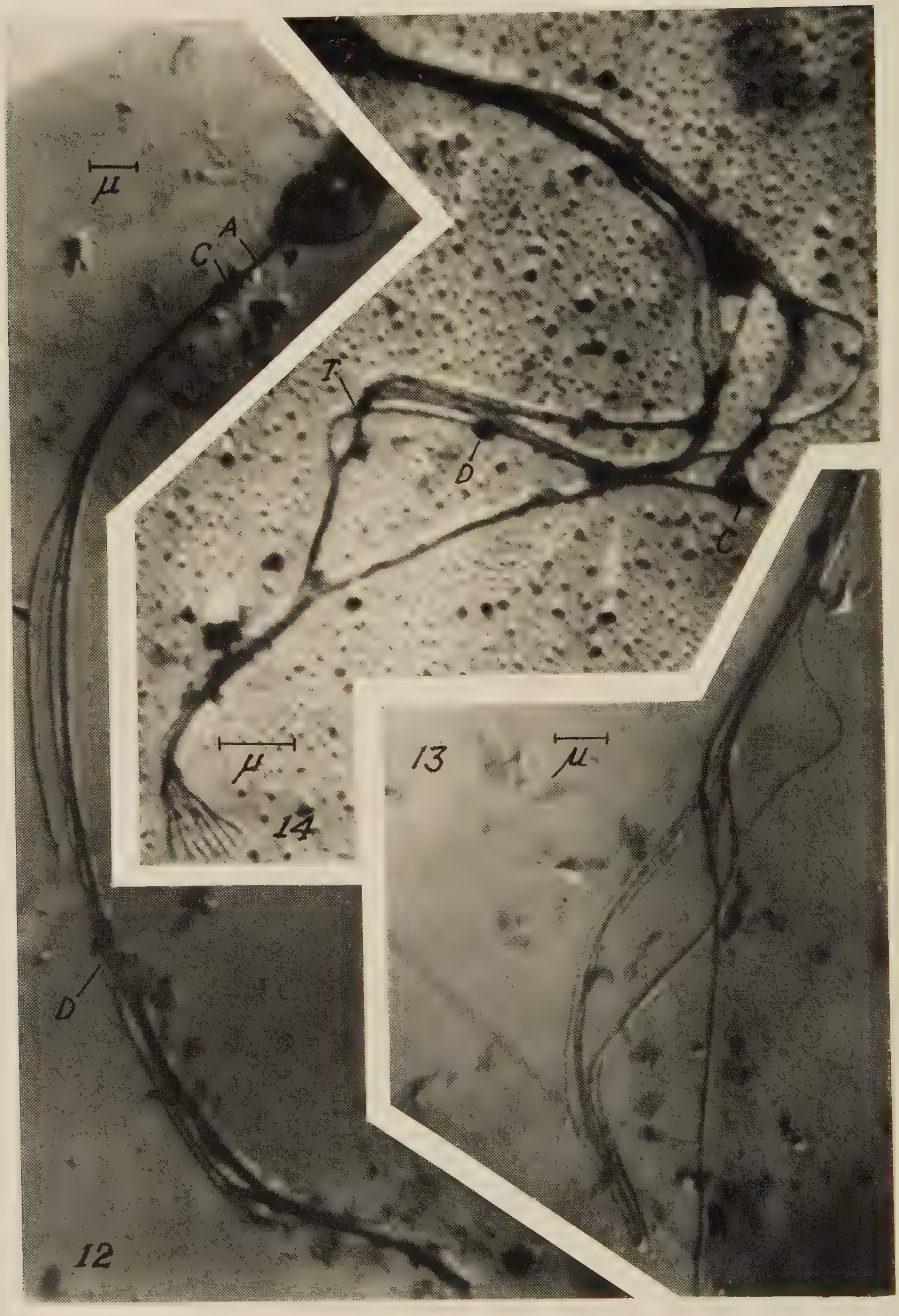
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As the masking effect of the basophilia, which renders *L. donovani* practically opaque to the electron beam, is removed by hydrolysis, more and more of the internal details become visible in the electron micrographs. The effect of a shorter period of hydrolysis is first noticed in the posterior part of the body which is the thinnest part in the whole cell. After prolonged hydrolysis for 30 to 40 minutes, the entire body becomes thin enough for the transmission of the electrons but the shape of the flagellate remains intact. The method of hydrolysis has therefore a decided advantage over the method of cell disruption by osmosis in distilled water in which the shapes are greatly distorted (7).

Another effect of hydrolysis is the dissolution of binding material that holds together the individual fibrils to form the composite flagellum. This reveals the inner core (A) of the flagellum (Fig. 12). The core measures about 1400 Å compared with the average width of 2200 Å of the untreated flagellum of *L. donovani*. The difference of 800 Å accounts for the protoplasmic sheath and the binding matrix. The diameter of the inner core of the composite flagellum (1400 Å) compares favourably with the average width of 450 Å of individual fibrils, if we consider the nine fibrils to be closely packed together.

The fibrils observed in *Leishmania donovani* compare favourably in number and width with those found in different groups of other related flagellates e.g. *Trypanosoma evansi* (6) and in non-related flagellates e.g. *Trichonympha sp.* (9), *Euglena gracilis* (8), *Polytomella magna* (1) etc. The intermediate fibrils are probably binding in function. They untwine first during the process of resolution of the composite flagellum into the component bundles. The whole bundle of fibrils occurring in a twisted rope like arrangement gives rise to the composite flagellum.

SUMMARY

1. The flagellate form of *Leishmania donovani* observed under the phase microscope shows that in the living condition the nuclear region is optically lighter then the rest of the cell with the exception of vacuole.

Fig. 12. The protoplasmic matrix of the splitted flagellum is found adhering at the places C and D while the central core is exposed at A. Magnification: 6000 ×.
Fig. 13. Nine distinct fibrils arranged in a tuft and slightly twisted are clearly visible. Magnification: 7000 ×.
Fig. 14. Flagellum splitted into nine fibrils in a very characteristic manner, with the proximal part still intact and the fraying effect remarkable at the distant end, giving rise to a finger tip appearance. Magnification: 10,500 ×.

2. In direct electron micrographs of OsO_4 fixed specimens, the whole body of the flagellate appears uniformly dark and no internal details are visible.

3. A method of hydrolysis has been described which makes the flagellates suitably thin for electron microscopic study of detailed morphology.

4. This method reveals clearly the nucleus with an outer dark margin, a central karyosome, the parabasal body and longitudinal myoneme fibrils arranged in inter-connected bundles on the periplast.

5. The flagellum is found to resolve first into two bundles with two intermediate fibrils and finally into a maximum number of nine fibrils with an average width of 450 Å.

The authors are indebted to Dr. P. C. Sen-Gupta, School of Tropical Medicine, Calcutta for ungrudging supply of all *Leishmania* cultures and to Prof. M. N. Saha, F.R.S. for his encouragement and interest in this work.

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STUDIES ON THE CYTOCHEMISTRY OF ALKALINE PHOSPHATASE IN VARIOUS BACTERIA

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The staining technique for alkaline phosphatase developed by Gomori (4) has given rise to extensive investigations on the localization of this group of enzymes in animal and plant tissues. Bayliss *et al.* (1) applied this technique to bacteria and reported discrete intracellular localizations of alkaline phosphatase in several rod-like bacteria. In the light of more recent innovations in methods for the cytochemical localization of alkaline phosphatase (5) and of newer concepts concerning the structural organization of the bacterial cytoplasm (9), further study of this enzyme in bacteria is timely.

This paper presents a correlated study of alkaline phosphatase in bacteria as determined chemically (expressing the activity of the enzyme), and cytochemically, under various conditions. The localization of the stain is also described.

MATERIALS AND METHODS

Organisms studied. Bacillus brevis, Bacillus cereus, Bacillus cereus var. *mycoides, Bacillus coagulans, Bacillus firmus, Bacillus lentus, Bacillus megaterium, Bacillus pumilus, Bacillus subtilis, Sarcina lutea, Micrococcus pyogenes* var. *aureus, Serratia marcescens, Pseudomonas aeruginosa, Alkaligenes faecalis, Proteus vulgaris, Aerobacter aerogenes, Escherichia coli* (laboratory strain), and *Escherichia coli* (strain K-12). All but the last organism were from the stock collection of the Department of Bacteriology, University of Kansas. The culture of *E. coli* K-12 was obtained from the Department of Microbiology, School of Medicine, University of Pennsylvania.

Medium and substrate solutions. Bacto-Tryptose agar (Difco) was used in all experiments. Phosphatase determinations were made by measuring the amount of phosphate liberated from a substrate of the following composition: beta-sodium glycerophosphate 0.5 gm, sodium diethylbarbiturate 0.5 gm, distilled water q.s. 100.00 ml; adjusted to pH 9.4–9.6. "Tris" buffer (Tris(hydroxymethyl) amino

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[1] Sigma Chemical Company, Saint Louis, Missouri.

methane)[1] 0.05 molar, was tested as a substitute for the barbiturate, with identical results.

Chemical determinations. Bacterial suspensions were standardized for turbidity with an Evelyn Photoelectric Colorimeter, using a 420 mμ filter. The liberated phosphate was determined with the same instrument and a 660 mμ filter, according to the procedure of Fiske and Subbarrow (3).

Staining procedure. The staining reaction was carried out as follows. Thin smears of the cultures were made on slides or cover slips, and air-dried. Whenever parallel determinations were necessary, several smears were made on the same slide. The preparations were immersed in the substrate solution, to which 0.05 molar (final concentration) calcium chloride was added, for periods varying from one-half to four hours. They were kept at 37° C. At the conclusion of the incubation time the slides were immersed in a 2 per cent cobalt nitrate solution for five minutes, rinsed with distilled water, and treated for one minute with ammonium sulfide reagent (light yellow). They were rinsed with water, dehydrated with graded alcohols, cleared with xylol, and mounted in Technicon[1] mounting medium.

The azo dye method of Menten *et al.* (7), as modified by Gomori (5), was performed with the use of tetrazotized dianiside (Naphthanyl Diazo Blue B).[2]

Controls for all of the experiments were set up in an identical manner, but with the glycerophosphate omitted. Controls of the cytochemical tests were either devoid of stain or revealed only a weak staining reaction without any discernible localization. Dipping the slides in boiling water for ten seconds stopped the reaction completely. The values of the phosphate determinations of the controls were subtracted from the experimental values. The figures for phosphatase activity were calculated from the slopes of curves obtained by performing at least four determinations over time periods of no more than twenty hours.

RESULTS

The effect of acetone fixation and pretreatment with citrate buffer. Acetone fixation of tissues previous to phosphatase staining is commonly employed. In order to determine the effect of this reagent on the enzyme under study, suspensions of *B. subtilis* were treated with absolute acetone at various temperatures. After washing with saline solutions, the suspensions were placed in the substrate, and the liberated phosphate was determined. A suspension treated with acetone at 37° C for ten minutes showed an increase in activity of 25 per cent over the untreated control. Treatment at −20° C for ten minutes gave a 52 per cent increase, and thirty minutes at the same temperature gave a 63 per cent increase. In slide preparations none of these treatments resulted in any discernible change of intensity or localization of the staining reaction as compared with unfixed preparations.

Acid citrate buffer has been employed in the past for the removal of pre-

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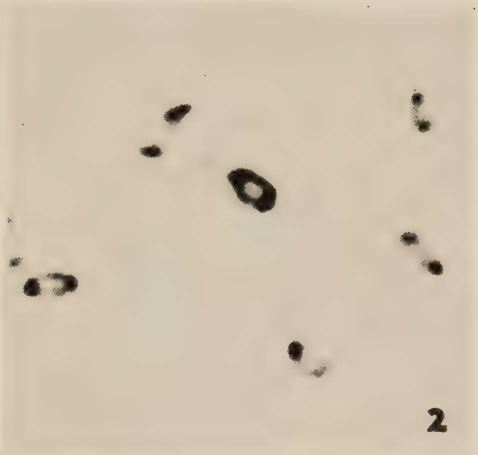
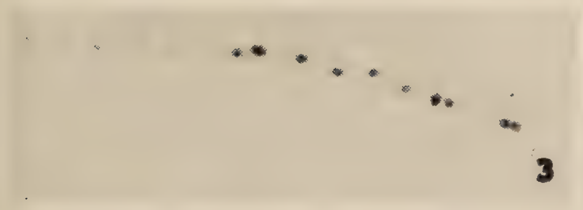
[1] Technicon Mounting Medium, Technicon Chemical Company, Inc., New York 51, N.Y.

[2] Dajac Laboratories, Leominster, Massachusetts.





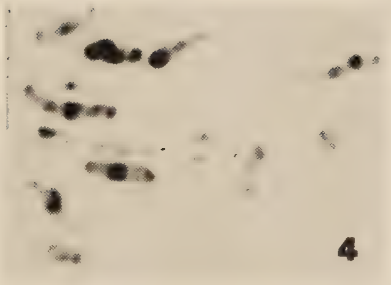


Fig. 1 – *A. aerogenes.*
Fig. 2 – *E. coli* (laboratory strain).
Fig. 3 – *B. cereus* var. *mycoides.*
Fig. 4 – *E. coli* K-12.
Figs. 1, 2, and 3 — 24-hour cultures on agar containing M/100 $MnSO_4$. Four-hour incubation in glycerophosphate substrate.
Fig. 4 — $1^1/_2$-hour culture on agar containing M/1000 $MnSO_4$. Four-hour incubation in glycerophosphate substrate. Photomicrographs were enlarged to 4850 ×.

formed calcium (1). Suspensions of *B. subtilis* were treated with 0.1 molar citrate buffer at pH 4.7. After fifteen minutes at room temperature, the enzyme activity dropped 39 per cent.

The effect of pH. The optimum pH zone for the alkaline phosphatase of *B. subtilis* was found to be between pH 8.8 and 9.2. The intensity of the staining reaction of this and seven other organisms was considerably weaker when the pH of the substrate was below pH 8.8. This decrease in intensity, probably due to the greater solubility of the calcium phosphate formed in the reaction, was not accompanied by any observable change in the localization of the stain.

Influence of the calcium concentration. E. coli K-12 was placed in substrates containing amounts of calcium varying from 0.1 molar to 0.005 molar. A diminution of the intensity of the stain proportional to the decrease in calcium concentration was found below the 0.05 molar level. The localization of the stain was identical at all concentrations giving perceptible staining.

Influence of the addition of metals to the culture medium. It has been reported (1) that staining for alkaline phosphatase is increased in intensity by the addition of manganese or magnesium and alanine to the culture medium of various organisms.

In the present experiments 2.77 grams of $MnSO_4 \cdot 7H_2O$ and 1.5 grams of alanine were added to one liter of medium before sterilization. The original concentration of manganese, 0.01 molar, was considerably reduced by the formation of a heavy precipitate during sterilization. This medium was inhibitory to *B. cereus, B. coagulans, B. firmus, B. lentus, P. vulgaris* and *S. lutea.*

The following organisms showed increases in staining after growth on this medium: *A. aerogenes, A. faecalis, P. aeruginosa, E. coli* (laboratory strain and K-12), *S. marcescens, B. brevis, B. megaterium, B. cereus* var. *mycoides, B. pumilus* and *B. subtilis.* The extent of the increase in the staining reaction after various transfers on manganese-supplemented agar varied greatly. *E. coli* and *B. megaterium* needed only one transfer to give strong staining reactions, whereas *B. brevis* and *B. subtilis* required four or five subcultures.

Cell suspensions of *B. subtilis* were used to study quantitatively the influence of five divalent cations added to the medium in concentrations ranging from 10^{-3} to 10^{-5} molar. Ferrous, cupric and magnesium sulfates did not change the levels of alkaline phosphatase activity significantly. Zinc sulfate produced a 30 per cent drop of activity at a concentration of 10^{-3} molar, a 20 per cent drop of activity at 10^{-4}, and no significant change at 10^{-5} molar. Manganese sulfate gave 25 per cent, 18 per cent and 0 per cent increases in activity with 10^{-3}, 10^{-4} and 10^{-5} molar concentrations, respectively. As already stated, a marked increase in the staining reaction was found in cells grown on manganese, whereas iron, copper and magnesium had no influence. Zinc proved to be inhibitory.

Localization of the stain. Young cultures of *E. coli* K-12 (one and one-half to three hours old) showed darkly-stained granules in the cytoplasm with cell walls occasionally stained. The entire cytoplasm appeared lightly stained in many cells. The granules, usually one or two per cell, ranged in size from the same diameter as that of the cell to the limit of vision. Their position was either central or polar. The small granules were found at the periphery of the cell (Fig. 4). The nuclear area (central in uninucleate cells, subterminal in binucleate cells) was never stained. Parallel staining with triphenyl tetrazolium chloride after the procedure of Mudd *et al.* (8) revealed the formation of a red formazan in the same granules. The laboratory strain of *E. coli* showed two large granules per cell, usually at the poles. Cell walls were usually stained in this strain (Fig. 2). Old cells of these two organisms (twenty-four hour cultures) were more irregular in staining, some being unstained and some showing large stained granules. The latter type of cells elicited a strong staining reaction in thirty minutes, whereas the young cells needed two to four hours. Young cells of *E. coli* K-12 stained in a fashion similar to that described for the Gomori method when treated with the azo dye technique. They required at least two-hours incubation at room temperature. The staining bath was changed every fifteen minutes.

Twenty-four hour cultures of *B. subtilis, B. brevis, B. cereus* var. *mycoides* (Fig. 3), *A. aerogenes* (Fig. 1), *P. aeruginosa,* and *S. marcescens* showed staining in bipolar granules.

Other organisms, including *B. pumilus, B. megaterium* and *M. pyogenes* var. *aureus,* showed irregular staining of the cell protoplasm and the cell wall. *B. megaterium* showed so much variation, even within a single microscopic field, that no cytological interpretation was possible.

DISCUSSION

The problems posed by performing the Gomori reaction in bacteria are to a certain extent *sui generis.* In tissue sections artifactual nuclear localizations are found, according to Gomori (5), when the pH is lower than pH 9.4 and the calcium concentration less than 0.05 molar. The fact that this phenomenon did not occur in bacterial preparations under either condition may be due to the presence of cell envelopes which remain intact during the course of the procedure employed, or to a higher density of cytoplasmic constituents.

Macary (6) found that the metachromatic granules of Coryne bacteria take up cobalt ions and reveal staining after treatment with sulfide. This was not found in those organisms which were devoid of metachromasy. The staining was interpreted as being due to the presence of meta- or polyphosphates in the metachromatic granules. The organisms used in the present work never showed any appreciable staining in the control preparations which were treated in the same fashion after incubation in substrate-free buffer. Ebel (2) found no measureable amounts of polyphosphates in Staphylococci, colon bacilli or anthrax bacilli. Since none of the above artefacts was found, it is felt that the cobalt sulfide staining procedure which was employed here is a useful tool in cytochemical studies of certain bacteria.

The mechanism of the observed activation of the enzyme by manganese remains unexplained. The quantitative data did not reveal increases in alkaline phosphatase activity in cells grown on manganese-supplemented agar sufficient to account for the greatly increased intensity of staining.

The localization of the stain in cytoplasmic granules was in accordance with the findings of Mudd *et al.* (8), who used a variety of mitochondrial stains. This was particularly marked in the case of *E. coli* K-12. In addition, staining of the cell wall and of the entire cytoplasm indicates that alkaline phosphatase in the organisms studied is not localized in one type of structure only. Definitive evidence must await some new approach, perhaps cell fractionation studies.

SUMMARY

The Gomori staining reaction for alkaline phosphatase was carried out in several bacteria. Fixation with acetone did not interfere with the staining reaction, whereas pretreatment with acid citrate buffer proved inhibitory to the enzyme. The optimum pH zone of the enzyme in *B. subtilis* (pH 8.8–9.2) was found to be close to that at which the staining reaction was carried out (pH 9.4). The intensity of staining was proportional to the concentration of calcium in the incubating mixture. No concomitant change in localization was found. The addition of manganese to the culture medium significantly increased the staining intensity in ten species. The localization of the stain in seven species was in cytoplasmic granules which appeared to correspond to those described as mitochondria by previous authors. Staining of cell walls and of the entire cytoplasm was also found.

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THE DEOXYRIBONUCLEIC ACID CONTENT OF INDIVIDUAL RAT CELL NUCLEI

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In 1948 Boivin, Vendrely and Vendrely (4) suggested that in any species all somatic nuclei might contain the same amount of deoxyribonucleic acid (DNA) and that the gamete nuclei, being haploid, might contain half this amount. The view that DNA might be a constant component of the chromosomes was also put forward independently by Mirsky and Ris in 1949 (19). Attempts have been made to test this hypothesis experimentally, both by gross chemical analysis of counted suspensions of cells and isolated nuclei and by cytophotometric measurements on individual nuclei (42). The results so far obtained have not been sufficiently definite to justify either the complete acceptance or rejection of the hypothesis.

In this connection rat cell nuclei are of particular interest. Chemical analysis of counted suspensions of isolated nuclei has shown that in this species kidney, spleen, lung, small intestine, thymus, salivary gland, pancreas, leucocytes and bone marrow all have approximately the same average DNA content per nucleus (40) but that the corresponding value for liver is considerably higher (8, 16, 40). These observations have been regarded as supporting the hypothesis that in the rat the DNA content per set of chromosomes is constant for resting interphase nuclei, the anomalously high value obtained for liver being attributed to the presence, established by purely histological methods, of a considerable proportion of tetraploid and octoploid cells (2, 38, 3, 18). This interpretation is partly supported by evidence that the average DNA content of adult rat liver nuclei does not appear to vary with sex, strain or body-weight, or to be affected by short-term dietary treatments (40). Moreover, the fact that the average DNA content of liver nuclei is lower in young animals than in adults (40, 37, 7) is in agreement with the histological observation that growth of the liver is accompanied by an increase in the proportion of polyploid nuclei (18).

Chemical analyses of counted suspensions of isolated nuclei, however, cannot by themselves constitute an adequate test of the hypothesis, since they can yield no information on possible variations between individual nuclei within the same tissue. We have therefore estimated the relative DNA contents of individual nuclei in a number of rat tissues in an attempt to answer the following questions: —

1. Does the average DNA content of diploid nuclei vary from one tissue to another?

2. Are the DNA contents of haploid, diploid, tetraploid, and octoploid nuclei in the ratios 1:2:4:8?

3. What, if any, is the biological variation in DNA content of individual nuclei of the same "ploidy" and within the same tissue?

The methods at present available for estimating the relative DNA contents of individual nuclei are: —

i) Measurement of optical extinction after Feulgen staining (34, 27, 39, 16, 22, 43, 6).
ii) Similar measurements after staining with methyl green (31, 11, 13, 12, 6).
iii) Measurements of the optical extinction of the unstained nuclei in ultra violet light at about 260 mμ (the absorption maximum of the nucleic acids) after removal of ribonucleic acid (15, 14, 6).

Method i) was chosen for the present investigation since it avoids the technical difficulties associated with ultraviolet microphotometry and since in this laboratory it has proved to be more satisfactory than method ii) (6). As the Feulgen reaction, properly applied, does not stain cytoplasm, method i) can be applied to nuclei either in tissue sections or after isolation (34). The use of tissue sections makes possible the classification of each measured nucleus according to cell type but introduces the danger of measuring nuclei which have been "chipped" by the microtome blade (21). It was therefore decided in the present instance to carry out measurements only on smear preparations of isolated nuclei.

EXPERIMENTAL

Animals. Adult male albino rats weighing 200–300 g and fed *ad libitum* on "rat cake" (Levers Cattlefoods, Ltd) were used. In the experiments on tumour tissue carcinogenesis was induced by administration of a diet containing 0.06 per cent of p-dimethylaminoazobenzene (40). Where partial hepatectomy (removal of median and left lateral lobes) was carried out, the method of Higgins and Anderson (9) was used. The animals were killed by exsanguination under ether anaesthesia and the tissues rapidly removed and stored in solid CO_2 until required.

Preparation of nuclei. Nuclei were prepared as described by Frazer and Davidson

(6), the preparations consisting of free nuclei contaminated to some extent with cytoplasmic debris and unbroken whole cells. Pancreas preparations, however, contained only a few free nuclei together with a large number of whole cells. Since under the conditions of the Feulgen reaction the cytoplasmic material did not stain, its presence was disregarded. Thin films of the preparations were made on microscope slides and allowed to dry. Rat spermatozoa were obtained by dividing the vas deferens close to the prostate gland, expressing a drop of fluid from the distal end on to a slide, spreading, and allowing the smear to dry. A film of normal kidney nuclei was made on each slide as a control. The slides were fixed in ethanol/acetic acid (3:1) for 30 minutes at room temperature, washed for two hours and dried.

Feulgen staining was carried out as described by Frazer and Davidson (6).

Cytophotometry. Cytophotometric measurements were made by the photographic method described by Frazer and Davidson (6). In this technique the whole field is evenly illuminated on the Kohler principle. In view of criticisms by Naora (22, 23, 24) that measurements under these conditions may be seriously influenced by lens glare (the so-called Schwarzschild-Villiger effect) one of the present series of measurements was repeated using the following modified lighting system: — The substage condenser was replaced by a 4 mm dry microscope objective (Leitz), the specimen being mounted on an extra-thin slide (ideally, a cover glass should be used). In front of the lamp condenser was placed a metal plate having two 1 mm circular apertures, 3 mm apart. The substage objective was adjusted to provide an image of these apertures in the plane of the specimen, which was positioned so that the image of one aperture was concentric with the nucleus to be measured, while the other occupied a closely adjacent area of clear background. Thus only the necessary areas of the slide were illuminated and the effect of glare was greatly reduced, although not eliminated completely (22, 23, 24). Single photographic exposures were made and densitometry carried out as previously described (6). The results obtained by this "two-spot" technique will be compared with those found for the same specimens by the "whole-field" illumination method.

RESULTS

The units of DNA content in which the results are expressed are entirely arbitrary and may vary in magnitude from one figure to another.

Normal liver. The results obtained in an experiment in which the DNA contents of 116 kidney and 479 liver nuclei were measured are shown in the form of frequency histograms in Fig. 1. The kidney nuclei form a single symmetrical peak extending from 270 to 720 units. Only 2 nuclei, with higher DNA contents, fall outside this group. The liver nuclei, on the other hand, fall into two or, more probably, three groups. The largest of these, which in accordance with convention may be termed Class I (39, 32), extends from 280 to about 700 or 800 units and clearly corresponds to the single kidney peak. The second main group (Class II) covers the range from 700 to 800 units to 1680 units. It may be presumed that the 2 kidney nuclei with DNA contents of about 1000 units also belong to this class. Finally, 7 liver nuclei with contents of 1920 to 2530 units presumably represent "Class III" nuclei. If the upper limit of the Class I kidney nuclei, 720 units, can be taken as the boundary between the Class I and Class II nuclei in the liver, the 479 liver nuclei measured may be divided into

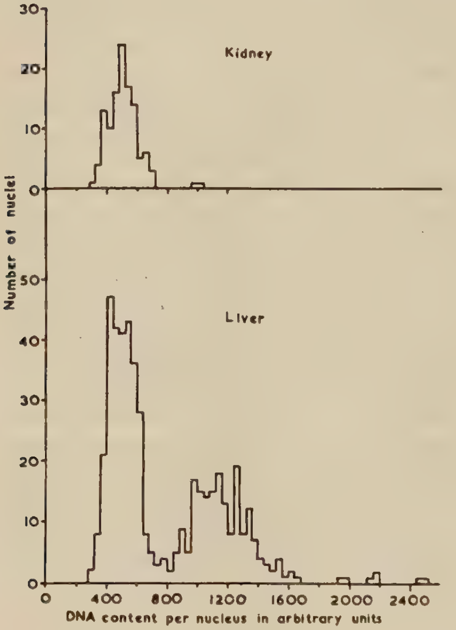

Fig. 1.

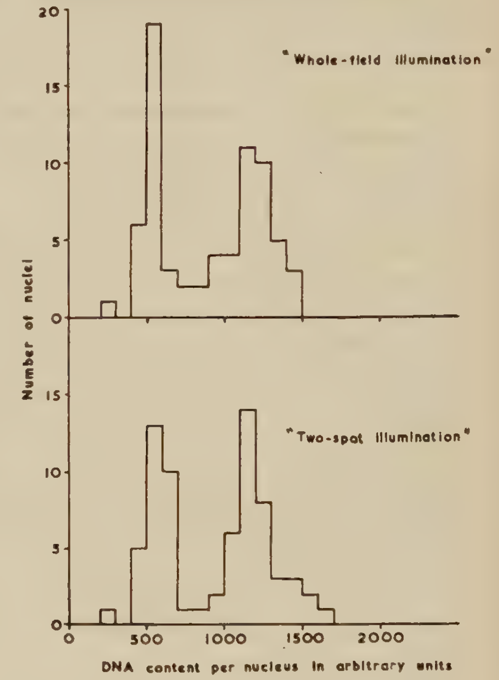

Fig. 2.

Fig. 1. Relative DNA contents of individual rat kidney and liver nuclei. *Upper chart:* 116 kidney nuclei, mean DNA content 509 units. *Lower chart:* 479 liver nuclei, mean DNA content 783 units. Fig. 2. Relative DNA contents of 70 rat liver nuclei measured (a) using "whole-field" illumination and (b) using "two-spot" illumination.

261 Class I nuclei, 58.7 per cent of the sample. DNA contents 280–720 units, mean = 502 units;

191 Class II nuclei, 39.9 per cent of the sample, DNA contents 720–1680 units, mean = 1145 units;

and 7 Class III nuclei, 1.46 per cent of the sample, DNA contents 1920–2520 units, mean = 2197 units.

The mean DNA content of the Class I kidney nuclei is 500 units, i.e. virtually the same as that of the Class I liver nuclei. Also, the mean DNA contents of the three classes of nuclei in the liver are approximately in the ratios 1 : 2 : 4. These observations agree with the findings of most workers who have investigated the relative DNA contents of individual nuclei in rat liver (34, 15, 16, 22).

Lens glare. Reference has already been made to the claim of Naora that lens glare may introduce serious errors into cytophotometry (22, 23, 24). Fig. 2 shows the results obtained by measuring the DNA contents of 70 liver nuclei (a) using normal "whole-field" illumination and (b) using "two-spot" illumination providing greatly reduced lens glare. It will be seen that the two methods give very similar results, and in particular, that the mean values for the Class I and Class II peaks are not appreciably increased when "two-spot" illumination is used, although the reduction of glare effects should produce such an increase if "whole-field" illumination did introduce serious errors of the sort described by Naora (22, 23, 24). These observations support the contention of Ornstein and Pollister (26) that, except under special circumstances, lens glare is not a major source of error in cytophotometry, and measurements made under "whole-field" illumination do not appear to be invalidated.

Pancreas and small intestine. Fig. 3 presents the results of an experiment in which the relative DNA contents of nuclei from kidney, pancreas and small intestine were measured. All the kidney nuclei measured, and all but one of the small intestine nuclei, clearly belong to Class I. The remaining intestine nucleus probably belongs to Class II. Pancreas appears to contain a considerable proportion of Class II nuclei. If 600 units, the upper limit of the Class I nuclei of kidney and small intestine, is taken as the boundary between Class I and Class II nuclei in pancreas, the 213 pancreas nuclei measured may be divided into

174 Class I nuclei, 81.7 per cent of the sample, DNA contents 240–600 units, mean = 439 units;
and 39 Class II nuclei, 18.3 per cent of the sample, DNA contents 620–1120 units, mean = 858 units.

As in liver, the mean DNA contents of Classes I and II are in the approximate ratio 1 : 2, and the mean DNA content of the Class I pancreas nuclei is very close to that found for the Class I nuclei of kidney (419 units) and small intestine (407 units).

Spermatozoa. Fig. 4 compares the DNA contents of rat sperm heads and rat kidney nuclei. The kidney nuclei all belong to Class I, with average DNA content of 337 units. The sperm heads similarly form a single group, with average DNA content of 195 units. The ratio, mean DNA content of sperm heads/mean DNA content of Class I nuclei, is thus 195/337 = 0.59 : 1.00, instead of 0.50 : 1.00 as predicted by Boivin, Vendrely and Vendrely (4). This discrepancy is not altogether surprising since the characteristic sickle shape of the rat sperm head, together with its relatively high optical density after staining, present unusual difficulties in accurate photometry by the present method.

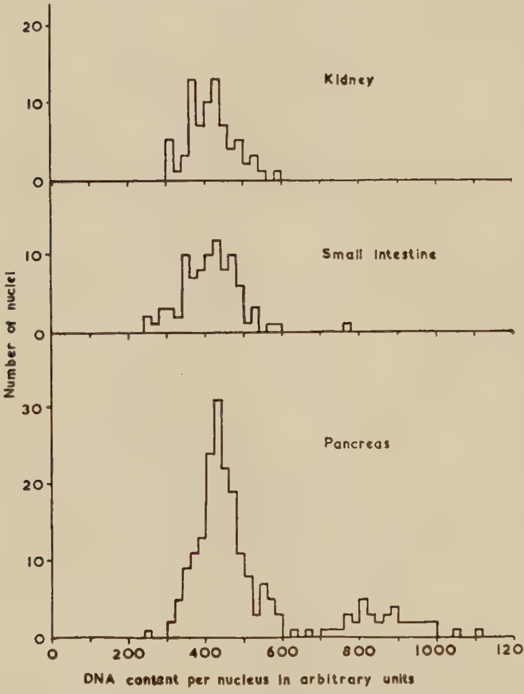

Fig. 3.

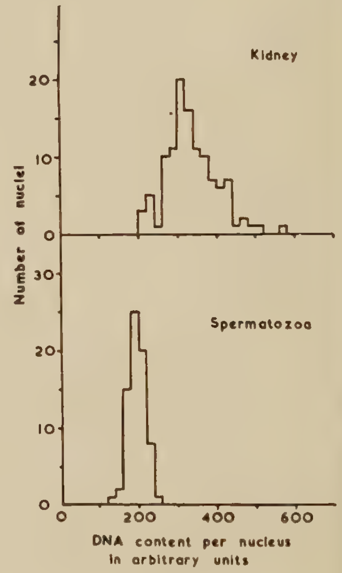

Fig. 4.

Fig. 3. Relative DNA contents of individual rat kidney, small intestine and pancreas nuclei. *Upper chart:* 75 kidney nuclei, mean DNA content 419 units. *Middle chart:* 88 small intestine nuclei, mean DNA content 411 units. *Lower chart:* 213 pancreas nuclei, mean DNA content 515 units.

Fig. 4. Relative DNA contents of individual rat sperm heads and kidney nuclei. *Upper chart:* 113 kidney nuclei, mean DNA content 337 units. *Lower chart:* 72 sperm heads, mean DNA content 195 units.

Tumour-bearing liver. Fig. 5 shows the relative DNA contents of nuclei isolated from the tumour-bearing liver of a rat maintained for 14 weeks on a carcinogenic diet. Of the 168 control kidney nuclei, 166 form a single peak extending from 160 to 500 units (mean = 358 units) and clearly belong to

Class I. The other two nuclei, with DNA contents of 720 to 800 units, belong presumably to Class II. The nuclei of the cancerous liver appear to fall into the same three classes as the nuclei of normal liver. If the upper limit of the Class I kidney nuclei (in this experiment 600 units) is chosen as the boundary between Class I and Class II in liver, the liver nuclei may be divided into

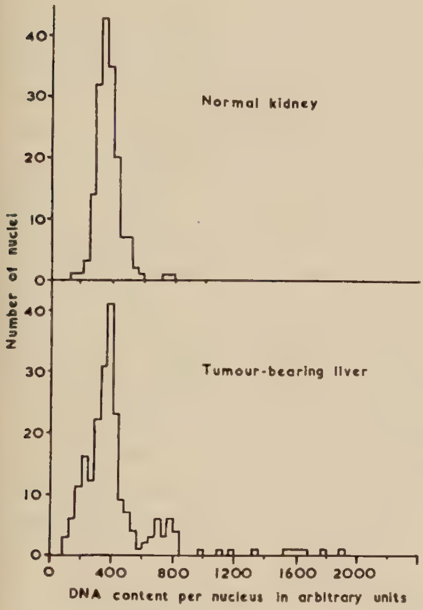

Fig. 5.

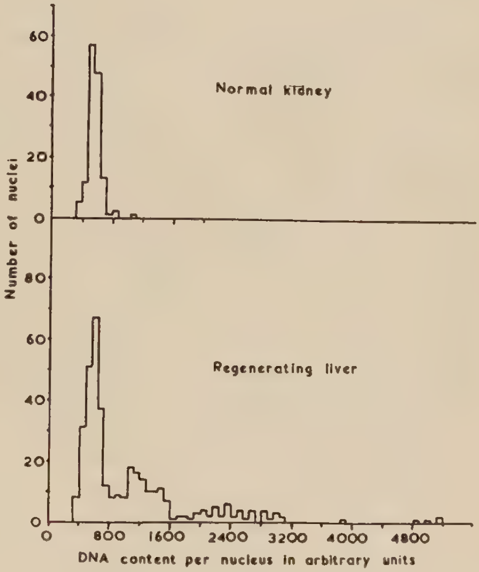

Fig. 6.

Fig. 5. Relative DNA contents of individual nuclei of rat kidney and tumour-bearing liver. *Upper chart:* 168 kidney nuclei, mean DNA content 360 units. *Lower chart:* 226 liver nuclei, mean DNA content 441 units.

Fig. 6. Relative DNA contents of individual nuclei of rat kidney and regenerating liver (48 hours after partial hepatectomy). *Upper chart:* 139 kidney nuclei, mean DNA content 560 units. *Lower chart:* 371 liver nuclei, mean DNA content 1053 units.

186 Class I nuclei, 83.7 per cent of the sample, DNA contents 80–600 units, mean = 336 units.

24 Class II nuclei, 10.8 per cent of the sample, DNA contents 600–840 units, mean = 733 units;

10 Class III nuclei, 4.5 per cent of the sample, DNA contents 960–1920 units, mean = 1468 units;

and 2 nuclei, 0.9 per cent of the sample, DNA contents 3120–3160 units, mean = 3140 units (presumably Class IV).

Once again the mean DNA contents of the different classes are approximately in the geometrical progression 1:2:4:8, and the mean DNA content for Class I liver nuclei is approximately the same as for Class I kidney nuclei.

Comparing Figs. 1 and 5, one sees that the carcinogen has increased very considerably the proportion of Class I nuclei in the liver (from 58.7 to 83.7 per cent) and has decreased the proportion of Class II nuclei (from 39.9 to 10.8 per cent), i.e. there is apparently a dilution of the Class II nuclei of normal liver tissue by Class I tumour nuclei. Other factors may also be involved (e.g. destruction of normal liver tissue due to compression by growing tumour nodules).

Regenerating liver. The DNA contents of nuclei isolated from liver undergoing compensatory hyperplasia (48 hours after partial hepatectomy) are shown in Fig. 6. All but one of the 139 control kidney nuclei form the usual symmetrical and fairly compact Class I peak extending from 320 to 880 units with a mean of 557 units, the exception (containing 1080 units) presumably belonging to Class II. The nuclei of regenerating liver, like those of tumour-bearing liver, fall into four classes. Taking the upper limit of the class I kidney nuclei, 880 units, as the boundary between Class I and Class II in regenerating liver, the liver nuclei may be divided into

214 Class I nuclei, 57.7 per cent of the sample, DNA contents 320–880 units, mean = 580 units;
103 Class II nuclei, 27.7 per cent of the sample, DNA contents 880–1600 units, mean = 1224 units;
49 Class III nuclei, 13.2 per cent of the sample, DNA contents 1600–3120 units, mean = 2373 units;
and 5 Class IV nuclei, 1.35 per cent of the sample, DNA contents 3840–5200 units, mean 4508 units.

Once again, the mean DNA contents for Classes I, II, III and IV are approximately in the ratio 1 : 2 : 4 : 8 and the mean DNA content for Class I liver nuclei is approximately the same as for Class I kidney nuclei.

If the pattern of results for regenerating liver is compared with that for normal liver, it is apparent that both contain the same three classes of nuclei, but in somewhat different proportions. In regeneration the proportion of Class II nuclei has fallen from 39.9 to 27.7 per cent and there has been a corresponding increase in the proportion of Class III nuclei from 1.46 to 13.2 per cent. A few nuclei which appear to belong to Class IV have also emerged. Presumably these changes are responsible for the marked but transient increase in average DNA content per nucleus during the first four days following partial hepatectomy, which has been reported by several authors (33, 41, 40).

Variation between nuclei of a single Class. A striking feature of the results shown in Figs. 1 to 6 is the wide scatter of values for individual nuclei of the same Class and within the same tissue. For example, the Class I kidney nuclei shown in Fig. 1 have apparent DNA contents ranging from 280 to 720 units with a mean of 500 units and a standard deviation of 87 units. The coefficient of variation for this group is therefore $87/500 \times 100 = 17.4$ per cent.

TABLE I

Analysis of variance to determine significance of variation in deoxyribonucleic acid (DNA) content between 24 rat kidney nuclei (4 estimations on each nucleus).

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	23	54 537	2 371.2	2.09
Between estimates	3	12 197	4 065.7	
Residual error.	69	78 197	1 133.3	
Total	95	144 931		

For $n_1 = 24$, $n_2 = 70$, $F = 2.07$ on the per cent significance level.

There is therefore a significant difference in DNA content between the individual nuclei. The component of the variance corresponding to the difference is $\frac{2\,371.2 - 1\,133.3}{4} = 309.5$. Since the grand mean for the 24 nuclei $= 365.3$, the coefficient of variation between nuclei $= \frac{\sqrt{309.5}}{365.3} = 4.8$ per cent.

In order to determine how far this wide scatter was attributable to experimental error and how far it might reflect a true biological variation, the DNA contents of 24 Class I kidney nuclei were estimated 4 times in the usual way and the figures obtained submitted to analysis of variance (Table I). The results of this analysis showed a significant difference in apparent DNA content between individual nuclei, with a coefficient of variation estimated to be 4.8 per cent. The real variation in DNA content from one Class I kidney nucleus to another may, in fact, be less than this estimate since some of the errors of the method cannot be eliminated by replicating measurements, e.g. the error introduced by the fact that, although the method is theoretically valid only for spherical or ellipsoidal nuclei in which the absorbing material is evenly distributed within the nucleus (25), it is being applied in the present instance to nuclei which only approximate in varying degrees to these theoretical requirements.

A similar statistical test was applied to Class I and Class II liver nuclei, the DNA contents of 59 normal liver nuclei being measured twice. When the means of the DNA values for each nucleus were drawn as a frequency

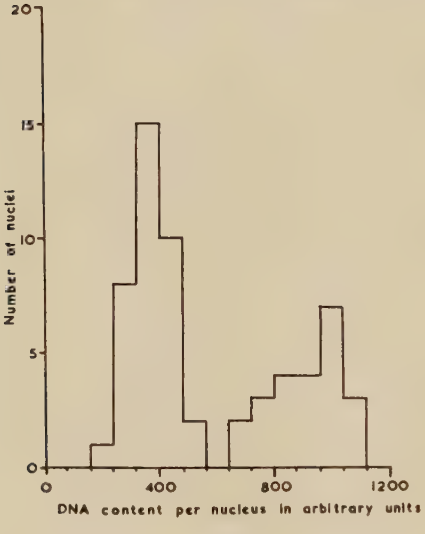

Fig. 7. Frequency histogram of DNA contents of 59 rat liver nuclei (mean of two estimations on each nucleus).

TABLE II

Analysis of variance to determine significance of variation in deoxyribonucleic acid (DNA) content between 36 Class I rat liver nuclei (2 estimations of each nucleus).

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	35	376 694	10 763	5.669
Between estimates	1	382	382	
Residual error.	35	66 451	1 899	
Total.	71	443 527		

For $n_1 = 30$, $n_2 = 34$, $F = 2.30$ on the per cent significance level.

There is therefore a significant difference in DNA content between the individual nuclei. The component of the variance corresponding to this difference is $\frac{10\,763 - 1\,899}{2} = 4432$. Since the grand mean for the 36 nuclei was 370.3, the coefficient of variation between nuclei $= \frac{\sqrt{4432}}{370.3} = 18$ per cent.

histogram (Fig. 7) they fell into two distinct peaks, from 160 to 560 units and from 680 to 1080 units. It was assumed that all the nuclei of the first peak belonged to Class I and all those of the second peak to Class II. The individual values contained in each peak were then subjected to analysis of variance (Tables II and III) and again a significant difference in apparent DNA content between the individual nuclei of each Class was found. The magnitude of the corresponding coefficients of variation was estimated to be 18 per cent for Class I and 11 per cent for Class II. Although these coefficients are considerably larger than that found for the kidney nuclei, this need not necessarily mean that the variation in DNA content between individual nuclei of the same class is greater in liver than in kidney, but may merely reflect the less uniform distribution of Feulgen stain commonly found in the liver nucleus as compared with the kidney nucleus.

TABLE III

Analysis of variance to determine significance of variation in deoxyribonucleic acid (DNA) content between 23 Class II rat liver nuclei (2 estimations on each nucleus).

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio "F"
Between nuclei	22	597 496	27 158	3.835
Between estimates	1	495	495	
Residual	22	155 788	7 081	
Total.	45	753 779		

For $n_1 = 24$, $n_2 = 22$, $F = 2.75$ on the 1 per cent significance level.

There is therefore a significant difference in DNA content between the individual nuclei. The component of the variance corresponding to this difference is $\frac{27\,158 - 7\,081}{2} = 10\,039$. Since the grand mean for the 23 nuclei $= 910.5$, the coefficient of variation between nuclei $= \frac{\sqrt{10\,039}}{910.5} = 11$ per cent.

DISCUSSION

The validity of the photometric comparisons made in the preceding section rests on the correctness of the assumption that after Feulgen-staining the amount of dye in a nucleus is proportional to the amount of DNA which that nucleus originally contained. Although it is generally agreed that Feulgen staining results from a reaction between fuchsin-sulphurous acid and the products of the hydrolysis of DNA, the chemistry of this reaction is still obscure (17, 29). Moreover, it has been shown that both the absorption spectrum of the dye produced and the absolute intensity of the staining are modified in presence of histones or other proteins (35, 20, 36). On theoretical grounds, therefore, it would be most unwise to regard the Feulgen reaction as quantitative. Nevertheless, it has been shown by Ris and Mirsky (34) that if nuclei isolated from different species are fixed and Feulgen-stained together on the same slide the average amount of dye per nucleus, measured cytophotometrically, is directly proportional to the average DNA content per nucleus for that species as determined by chemical analysis of counted suspensions of cells or isolated nuclei. Similar results have been obtained by the Leuchtenberger-Vendrely group (16). Moreover, those workers who have compared the different cytophotometric methods for estimating DNA have found that the Feulgen method gives essentially the same results as those in which methyl green staining is employed or in which the DNA content of unstained nuclei is estimated directly by ultraviolet absorption measurements (15, 16, 6). On empirical grounds, therefore, it appears probable that cytophotometric measurements following Feulgen-staining provide a fairly reliable means of estimating the relative DNA content of individual nuclei.

It is also implied in the method used in the present investigation that the nuclei measured constitute in each case a reasonably representative sample of the population of nuclei within the tissue under examination. It would

TABLE IV

Average DNA contents per nucleus of rat tissues relative to the average DNA content of the rat kidney nucleus.

Tissue	Average DNA content per nucleus in tissue ,, ,, ,, ,, ,, ,, kidney	
	Estimated in present experiments	Calculated from results of Thomson *et al.* (40)
Normal liver	1.54	1.40
Small intestine	0.98	1.14
Pancreas	1.23	1.09
Tumour-bearing liver . .	1.22	1.12
Regenerating liver (48 hours after hepatectomy) . .	1.88	1.93

clearly be a matter of some difficulty to determine how far this is in fact the case. In the present instance, however, a rough check can be obtained. The average DNA contents of the normal liver and kidney nuclei shown in Fig. 1 are 783 and 509 units, respectively. If these nuclei are representative samples from the tissues from which they were isolated, the average DNA contents per nucleus for liver and kidney should be in the ratio 783/509 = 1.54. But Thomson, Heagy, Hutchinson and Davidson (40) have found by chemical analysis of bulk nuclei isolated in citric acid that the average DNA contents per nucleus of these tissues are in the ratio 0.91/0.65 = 1.40. Similar ratios calculated from the data in Figs. 3, 5 and 6 are shown in Table IV. The moderately good agreement, for each tissue, between the results obtained by quite different methods appears to support the view that a reasonably representative sample of nuclei is being measured or analyzed in each case.

In considering the biological significance of the results obtained from the six somatic tissues investigated one must distinguish clearly between regenerating liver, in which growth is extremely rapid, and the other tissues (kidney, small intestine, pancreas, normal liver and tumour-bearing liver), in which growth is comparatively slow. The nuclei of the latter group appear to fall into four classes:— *Class I* nuclei, containing approximately twice as much DNA as the haploid sperm head, are the most numerous in all 5 tissues. Indeed, in kidney and small intestine virtually all the nuclei belong to this class. *Class II* nuclei, containing approximately twice as much DNA as Class I, are numerous in pancreas and in normal and tumour-bearing liver. They also occur, much more rarely, in kidney and intestine. *Class III* nuclei, containing four times as much DNA as Class I, are found in normal and tumour-bearing liver. Finally, *Class IV* nuclei, containing eight times as much DNA as Class I, occur in small numbers in tumour-bearing liver.

With the methods at present available it is not possible to estimate accurately the coefficient of variation in DNA content between individual nuclei within the same class. As has been shown above, however, it probably does not exceed 5 per cent for kidney nuclei and about 10–15 per cent for liver nuclei. The variations in average DNA content per nucleus between different tissues of the adult rat observed by a number of workers (5, 8, 16) appear to be attributable, not so much to variations in the characteristic DNA contents of the different classes of nuclei, as to variations in their relative proportions.

The fact that the average DNA contents of Classes I, II, III and IV are approximately in the ratio 1 : 2 : 4 : 8, and that Class I nuclei contain twice as much DNA as spermatozoa is in agreement with the theory that for any given species the DNA content per set of chromosomes is constant and that Classes I, II, III and IV represent respectively diploid, tetraploid, octoploid, and 16-ploid nuclei. It may be noted that Biesele's (3) estimates, based on chromosome counts, of the relative proportions of diploid, tetraploid and octoploid nuclei in rat liver are in fairly good agreement with the relative proportions of Class I, Class II and Class III nuclei found in this tissue in the present series of experiments, and that Jacobj's (38) measurements of nuclear volume suggest that about 20 per cent of the nuclei in rat pancreas are tetraploid.

Two groups of workers have, however, reported results quite inconsistent with these conclusions. Pasteels and Lison (27) investigated cytophotometrically the DNA contents of Feulgen-stained nuclei in a wide variety of tissues taken from a single adult rat. They found that, whereas in some tissues (e.g. kidney) all the nuclei contained approximately the same amount of DNA, in liver and pancreas the nuclei fell into three classes with DNA contents in the approximate ratio 1 : 2 : 4. In both tissues, however, the characteristic DNA contents of each class were approximately 30 per cent lower than the theoretical diploid, tetraploid, and octoploid values as estimated from measurements on the nuclei of other tissues. These results, which, if valid, would clearly exclude the concept of DNA as a constant element in the chromosomes, have never been confirmed by other workers. Pasteels and Lison (28) have recently reinvestigated the question by carrying out measurements on the Class II liver nuclei of a further three rats. In these animals they find, in agreement with all other workers who have made similar measurements (16, 15, 30, 6) that the average DNA content of such nuclei is not significantly different from the theoretical tetraploid amount (as estimated, in this case, from measurements on primary spermatocyte nuclei, which have been shown to contain twice as much DNA as somatic diploid nuclei (27, 39).

A third view of the relationship between the three classes of nuclei in rat liver has been put forward by Naora (22), who suggests that their characteristic DNA quotas are not in the geometrical progression 1 : 2 : 4 but in the arithmetical progression 9.5 : 13.5 : 17.5. This suggestion, however, rests on such a scanty basis of experimental evidence (only 4 Class II and 2 Class III nuclei appear to have been measured) that it hardly calls for detailed discussion at the present time.

The results obtained from regenerating liver are of particular interest in view of the high frequency of mitoses in this tissue. If the DNA content per set of chromosomes is in fact constant, it follows that mitosis must be associated with a rapid synthesis of DNA. It has been suggested by Swift and others (39, 1) on the basis of cytophotometric measurements on nuclei in rapidly growing tissues, that this synthesis precedes mitosis, i.e., that a nucleus which is about to enter prophase accumulates DNA until it contains twice its normal complement. The fact that the average DNA content per nucleus in regenerating liver is markedly increased above the normal resting level has therefore been explained by several authors as due to the high proportion of nuclei about to undergo mitosis in this way (30, 40). If this explanation were correct it would be expected that cytophotometric examination of regenerating liver should reveal the presence of large numbers of nuclei with DNA contents intermediate between the normal values for Classes I and II or II and III or III and IV. Examination of Fig. 6, however, shows that the results obtained from this tissue differ from those obtained from normal liver not so much in the frequency of intermediate values but rather in the relative proportions of the different classes of nuclei. In particular, the percentage of Class III nuclei in regenerating liver is much higher than in normal liver and the percentage of Class II nuclei correspondingly lower. Regenerating liver, unlike normal liver, also appears to contain a small percentage of Class IV nuclei. Two possible hypotheses might be put forward to explain these observations.

i) Some of the Class III nuclei of regenerating liver may not be true octoploid nuclei, but tetraploid nuclei which, in accordance with Swift's (39) theory, have doubled their DNA content in preparation for mitosis, and the Class IV nuclei may similarly be "pre-mitotic" octoploids. If this is the case, the large proportion of Class III and Class IV nuclei shown in Fig. 6 would indicate that there must be a considerable time interval between the completion of the pre-mitotic doubling of DNA content and the beginning of visible mitosis.

ii) Alternatively, it is conceivable that in regenerating liver DNA synthesis may coincide with, rather than precede, mitosis, and that the Class III and Class IV nuclei in this tissue may be true octoploid and 16-ploid nuclei. If this explanation is correct, it would appear that the process of regeneration is accompanied by a transitory increase in the relative proportion of such nuclei, corresponding to the transitory increase in average DNA content per nucleus.

A satisfactory answer to this problem must await the development of more accurate methods for the study of DNA synthesis in association with mitosis. The specific role, if any, of polyploid nuclei in liver and in pancreas also awaits elucidation.

SUMMARY

1. The relative deoxyribonucleic acid contents of individual nuclei in various rat tissues have been estimated cytophotometrically after Feulgen staining.

2. Evidence is presented that such estimations are not seriously affected by lens glare produced within the optical system of a normal microscope.

3. The nuclei of normal liver, kidney, small intestine and pancreas appear to fall into three classes with respect to their content of DNA. Class I nuclei, containing approximately twice as much DNA as the sperm heads, are in the majority in all four tissues, and virtually all the nuclei of kidney and small intestine belong to this class. Class II nuclei, containing approximately twice as much DNA as Class I, are numerous in pancreas and liver. A few Class III nuclei, containing approximately four times as much DNA as Class I, are also found in liver.

4. The tumour-bearing liver produced by prolonged administration of p-dimethylaminoazobenzene contains a much higher proportion of Class I nuclei than normal. Rapidly regenerating liver (48 hours after hepatectomy) contains an increased proportion of Class III nuclei. A few Class IV nuclei, containing approximately eight times as much DNA as Class I, are found in both regenerating liver and tumour-bearing liver.

5. The coefficient of variation in DNA content between individual nuclei of the same class probably does not exceed 5–15 per cent.

6. These observations are interpreted as supporting the view that in rat cell nuclei the DNA content per set of chromosomes is approximately constant.

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THE MOVEMENTS OF SPERMATOZOA OF BRACKEN (*PTERIDIUM AQUILINUM*)

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The assumption has been made repeatedly in the literature that fern spermatozoa are chemotactically attracted to mature female organs (archegonia) since (a) sperms in a suspension are seen to accumulate around the necks of mature archegonia; (b) sperms are attracted by certain organic substances, particularly malic acid as shown by Pfeffer (6), Bruchmann (1), Rothschild (7), and others and (c) most text books (e.g. Fritsch and Salisbury (4)) make the statement that some of these substances are present in archegonia. A thorough search of the literature has provided no clue as to where this statement originated.

The archegonium of bracken consists of a ventral portion embedded in the prothallus, and a neck portion. The neck, which projects above the surface of the prothallus, consists of a wall composed of four rows of cells; it encloses a single elongated neck canal-cell. The ventral portion contains a large egg-cell and the ventral canal-cell immediately above it. As the archegonium matures both canal-cells become disorganised and fill the canal with a mucilaginous substance. This swells on the admission of water, and, rupturing the neck at the apex, is discharged from the archegonium which is now ready for fertilization. Hanstein (5) was first to report that sperms accumulated in this extruded mucilage and this undoubtedly happens in bracken (Fig. 1).

As found at the start of the present work, the accumulation of sperms in the mucilage is due to the fact that on entering it they stop swimming. This change is irreversible since such sperms, on removal from the mucilage, never recover their original motility. Thus, sperms will accumulate in the mucilage without there being an attraction in the same way as flies would accumulate on a fly-paper. The object of the present investigation has been to find by quantitative methods whether or not there is an attraction of sperms to mature female organs.

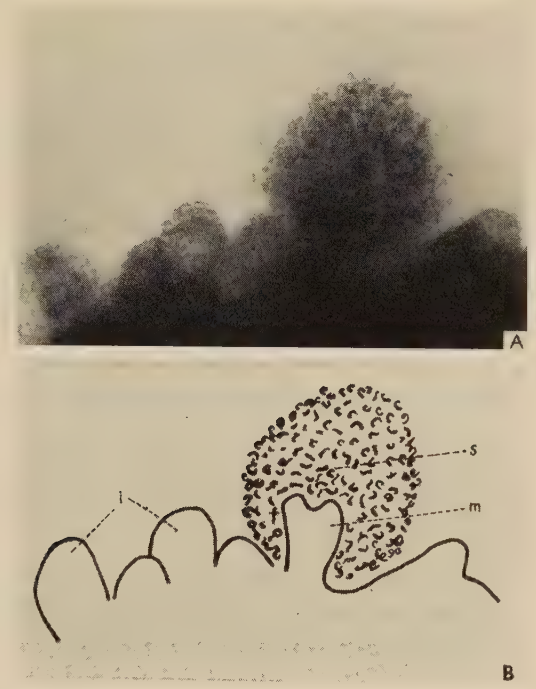

Fig. 1.
A. Photomicrograph of spherical mass of spermatozoa in the mucilage of a mature archegonium (× 60).
B. Diagrammatic representation of A.
s, the sperm mass.
m, open neck of mature archegonium.
i, closed necks of immature archegonia.

STATISTICAL APPROACH

The method of investigating the movements of spermatozoa in tap water was to mark out a grid on the surface of a glass slide using a diamond objective on a microscope fitted with mechanical stage. Squares on the grid had 0.5 mm sides and the grid was enclosed by strips of glass cut from a glass plate about 1 mm thick and fastened to the slide with wax (Fig. 2). The sperm suspension at 18° C was introduced into this compartment and the rectangular components x and y of the distance travelled by a sperm in a given time estimated under the dissecting microscope. The observations are summarised in Table I. Since the mean square displacement in unit time is constant then the movements of the sperm can be regarded as truly random and the behaviour of a suspension characterised by a diffusion

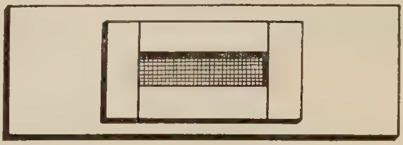

Fig. 2. Slide with grid enclosed by glass strips. Sperm suspensions are introduced into this compartment and the rectangular components of the distances travelled by sperms in given times measured on the grid.

TABLE I

Movement of spermatozoa in tap water at 18°C.

Time interval t	Mean and standard deviation for 100 measurements of co-ordinates of displacement				Mean of $\overline{x^2+y^2}$
	x	σ_x	y	σ_y	
5 sec.	0.38	0.22	0.41	0.24	0.44±0.03
10 ,, ...	0.53	0.40	0.54	0.37	0.86±0.08
20 ,, ...	0.76	0.49	0.72	0.56	1.77±0.10

The unit of x and y is the unit of the grid i.e. 0.5 mm.

coefficient $D = \frac{\overline{x^2+y^2}}{4t}$ (2, 3). From Table I, D averages 0.0055 (x and y in mm).

Suppose the initial concentration of such a suspension is C_0 (number per unit volume) and that organisms arriving on a fixed sphere of radius a are caught, then at times t from the start of the experiment the concentration at a point r away from the centre of the sphere will have fallen to a value C given by

$$C_0 - C = \frac{a\,C_0}{r}\left\{1 - \operatorname{erf}\frac{(r-a)}{2\sqrt{D\,t}}\right\}$$

$$\text{Then } -\frac{dc}{dr} = -\frac{a\,C_0}{r^2}\left\{1 - \operatorname{erf}\frac{(r-a)}{2\sqrt{D\,t}}\right\} - \frac{a\,C_0}{r}\cdot\frac{d}{dr}\cdot\operatorname{erf}\frac{(r-a)}{2\sqrt{D\,t}}$$

$$\text{i.e. } \frac{dc}{dr} = \frac{a\,C_0}{r^2}\left\{1 - \operatorname{erf}\frac{(r-a)}{2\sqrt{D\,t}}\right\} \frac{a\,C_0}{r\sqrt{\pi D t}}\cdot\exp\left[-\frac{(r-a)^2}{4\,D\,t}\right].$$

The rate of arrival on the surface is then $4\,\pi\,a^2 D\left(\frac{dc}{dr}\right)_a$

$$= 4\,\pi\,a^2\,D\,C_0\left(\frac{1}{a} + \frac{1}{\sqrt{\pi D t}}\right).$$

And the number which has arrived since time zero (start of the experiment) will be

$$4\,\pi\,a^2\,D\,C_0\left(\frac{t}{a} + 2\sqrt{\frac{t}{\pi\,D}}\right).$$

If in an actual experiment we find that the numbers of sperms arriving on the surface of the mucilage are greater than this then it can be taken that there is an attraction.

In a first experiment where the density of sperm suspension was 10^4/ml (estimated from haemocytometer counts), the initial rate of arrival at the mucilage was 1.2/sec. increasing steadily to a maximum of approximately 10/sec., the time taken to reach this maximum being about 5 minutes. Counting sperms at this stage presented a difficulty and the rate of 10/sec. was arrived at after independent estimates were made by colleagues. This rate of arrival was maintained for a further 5 minutes and thereafter steadily decreased to a minimum of 1 in 12 sec. The mucilage was then filled with sperms and it was at this stage that the photograph in Fig. 1 was taken. There was no further increase in this mass of sperms since those now arriving moved off again. The diameter of the mass then measured 0.5 mm.

In a second experiment the density of sperms was reduced to 10^3/ml, and in this case the maximum rate of arrival was 1.2/sec., a rate which could be counted accurately.

Putting in numerical values for a, D, and C_0 in the expression for the rate of arrival, we find that the term $\frac{1}{\sqrt{\pi D t}}$ may be neglected for observations taken later than 1 minute from the start of the experiment so that the rate expected after five minutes (when actual rate of arrival was at a maximum in the experiments) can be taken as $4\,\pi\, a\, D\, C_0$.

In the course of these experiments samples of sperm suspensions were withdrawn and diffusion coefficients calculated for sperms in these samples. These did not differ significantly from those found for sperms in tap water (Table II). In other words, the mobility of sperms in the neighbourhood of a mature archegonium was the same as that of sperms in tap water. It may also be mentioned that the number of sperms in the mucilage was small in comparison with the total number of sperms in the suspension, so that the change of the concentration of sperms in the suspension during the experiment could be neglected. The results of these experiments are summarised in Table II.

These results show that sperms are actually attracted to mature archegonia since their rate of accumulation in the mucilage is much greater than would be expected on the basis of a fly-catching mechanism. They also indicate that the attraction operates on the direction of motion rather than on its velocity. Rothschild (7) concluded that the effect of malic acid on bracken sperms was to change the direction of motion and not to increase its rate.

TABLE II

Rate of arrival of sperms at sphere of mucilage.

Experiment	Sperm density C_0	Diffusion coefficient (D)		Radius of sphere of mucilage a	Rate of arrival at mucilage	
		In tap water	During experiment		Expected ($=4\,\pi\,aDC_0$)	Measured
1	10^4/ml	0.0056	0.0051	0.25 mm	0.2 /sec.	10/sec.
2	10^3/ml	0.0055	0.0052	0.26 mm	0.02/sec.	1.2/sec.

QUALITATIVE APPROACH

In a further set of experiments the apparatus used was that shown in Fig. 3. Again strips of glass were fastened to a glass plate with wax. In compartment A was placed approximately 0.5 ml of a sperm suspension of density 10^3/ml shut off from channel CB by a rubber stopper at S. Compartments C and B and the connecting channel were filled with water. Into B was placed a prothallus with a mature archegonium and into C a prothallus with immature archegonia. These prothalli were previously rinsed in several changes of water to rid them of mature spermatozoa and also to examine them for mature archegonia. After 15 minutes (to allow any diffusion gradients to be set up) the stopper was removed. The flow of water was in the direction of A, the level of water in the channel CB being slightly higher than in AS. The field of the microscope was then focussed on the area at the junction of the two channels indicated by a circle in the diagram. Of

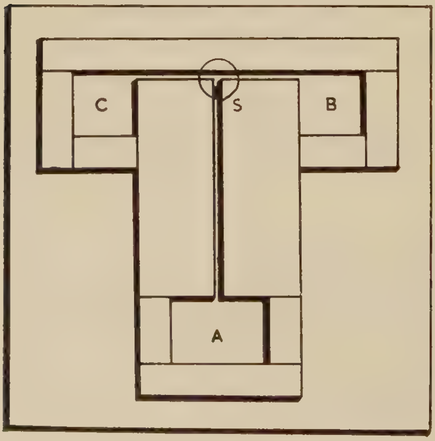

Fig. 3. Glass plate with channels and compartments A, B and C, enclosed by glass strips. *S*, rubber stopper. A sperm suspension is introduced into compartment A and water into the others. A prothallus with a mature archegonium is placed in B, and one with immature archegonia in C. The stopper is removed and the movements of sperms into B and C noted.

the first 20 sperms observed, 2 moved into the channel leading to C, the others having moved in the general direction of the right hand channel. After 5 minutes there were about 100 sperms in the right hand channel while 10 were counted in the left hand channel. After 10 minutes a mass of sperms had built up in the mucilage of the mature archegonium. At the same time there were no sperms to be seen in compartment C and 6 were counted in the left hand channel.

This type of experiment was repeated with several variations but the final result was the same in that the overwhelming majority of sperms swam towards the mature archegonium, giving strong evidence of an attraction presumably of a chemotactic nature.

To investigate the hypothesis of a substance released by the archegonium being responsible for this attraction, a few prothalli were each placed in 1 ml of water. After 15 minutes the water was taken up in a pipette from one of the prothalli showing a mature archegonium and left for about two hours, so that any sperms present would have died off. 0.1 ml of this liquid was introduced into compartment B (compartments B and C having been previously filled with water and A with a sperm suspension). After ten minutes the stopper at S was removed. Sperms were attracted to the solution, many more sperms moving into B than into C. When the experiment was repeated with the liquid diluted ten times sperms did not show a preference for compartment B but moved freely between C and B. Obviously, the concentration of the stimulating substance or substances cannot be much reduced (say more than 10^{-2}) without losing its activity. This finds a parallel with Pfeffer's (6) experiments in which sperms were not attracted to sodium malate solutions of less than 0.001 per cent.

BEHAVIOR OF SPERMATOZOA TOWARDS RUPTURED CELLS

Following the chance observation that spermatozoa are attracted to the broken cells of the prothallus a series of experiments was carried out. First it was shown that a single somatic cell of the prothallus, when punctured with a needle, was effective in attracting spermatozoa. Sperms were also shown to be attracted to the ruptured cells of the bracken sporophyte. The attraction was demonstrated for the ruptured cells of the leaves of *Senecio vulgaris, Poa annua, Fuchsia* and *Taraxacum*, as well as for cells of the fruit fly *Drosophila melanogaster*, an annelid and a mollusc.

Thus the attraction of sperms to archegonia is not specific but includes the cells of a variety of plants and animals, provided these cells are ruptured. Since the archegonium is able to bring about the disintegration of the neck cells, this property alone might account for the attraction, the hypothesis being that sperms are attracted to a substance or substances common to the cells of plants, animals and neck cells of archegonia. A simple organic acid would be such a substance. It is also possible that the neck cells of the archegonium contain a specific substance which attracts sperms and which is not to be found in other cells. However this latter hypothesis is the more unlikely of the two.

DISCUSSION

Rothschild (7) investigating the block to polyspermy in sea urchin eggs estimated the number of sperm-egg collisions by assuming that a suspension of spermatozoa could be treated as an assemblage of gas molecules in which the displacement is proportional to time. In the case of fern spermatozoa this assumption is erroneous (Rothschild himself expressed doubt as to its validity (7)), the square of the displacement being proportional to time. However it was his quantitative approach to the problem which stimulated the present investigation.

Both the simple statistical and experimental techniques which have been described here seem to be applicable to animal sperms.

SUMMARY

1. The movement of bracken sperms in tap water is random, the mean square displacement being proportional to the time.

2. It is known that when sperms arrive at the mucilage at the necks of mature archegonia they become immobilised; it has been found in the present work that this immobilisation is irreversible.

3. Sperms are attracted to mature archegonia since the number of sperms arriving in unit time at these archegonia is enormously greater than the number expected to arrive by chance. This increase in the rate of arrival is not due to an increase in the diffusivity but, presumably, to a superposed directed attraction.

4. When sperms are presented with two routes along which they may swim they take preferentially the route which leads to a mature archegonium, indicating that they are attracted in that direction. Sperms are also attracted to the water in which a mature archegonium has been previously immersed.

5. The attraction of sperms is not specific for mature archegonia since

sperms are attracted to *ruptured* cells of prothalli and sporophytes. Ruptured cells of a variety of animals and plants are also effective.

6. It is suggested that, since the maturation of the archegonium involves the rupture of two neck cells, this process alone might account for the attraction.

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PROPERTIES OF ISOLATED CARROT CHROMOPLASTS

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The crystalline shapes of most chromoplasts of carrots and their "dissolution" after treatment with organic solvents during microscopic observation of carrot slices has often led to the confusion of the chromoplasts with crystals of carotene. However, the plastid nature of the bodies has become more apparent through recent studies indicating that a "stroma", in part submicroscopic, of granular, fibrillar and lamellar structure is probably responsible for the shapes (20, 21). This interpretation is further supported by the observation that some chromoplasts of carrots have filamentous, round and spiral shapes, and that these, as well as the polygonal shapes of the chromoplasts are duplicated by colorless areas which appear in the moving cytoplasm of living xylem cells of carrots and represent perhaps the precursors of the chromoplasts (22). The preliminary chemical analysis of isolated chromoplasts reported in the following will confirm the cytoplasmic nature of the carotene carriers of carrots by revealing their content of typical cytoplasmic constituents such as ribonucleic acid and phospholipids.

Due to the strong tendency of chromoplasts to adsorb cytoplasmic granules during most steps of purification and to disintegrate into fragments of microscopic and submicroscopic sizes, the isolation of the chromoplasts poses considerable difficulties. Chromoplast fractions containing 2.4 per cent carotene previously separated from carrot juice by Vecher (23, 24) and those containing 4.8 per cent carotene by the writer (19) were still much too impure to serve as a basis for biochemical analysis. By an improved method which will be described in a later communication, it was possible to obtain practically pure chromoplasts free from cytoplasmic granules. Fig. 1 shows a preparation consisting mostly of whole chromoplasts of polygonal shapes, while other preparations used in this study contained in addition to whole chromoplasts large fragments of them as they may be seen in Fig. 2 of a recent communication (21). The composition of the large fragments probably does not differ much from that of whole chromoplasts. The small,

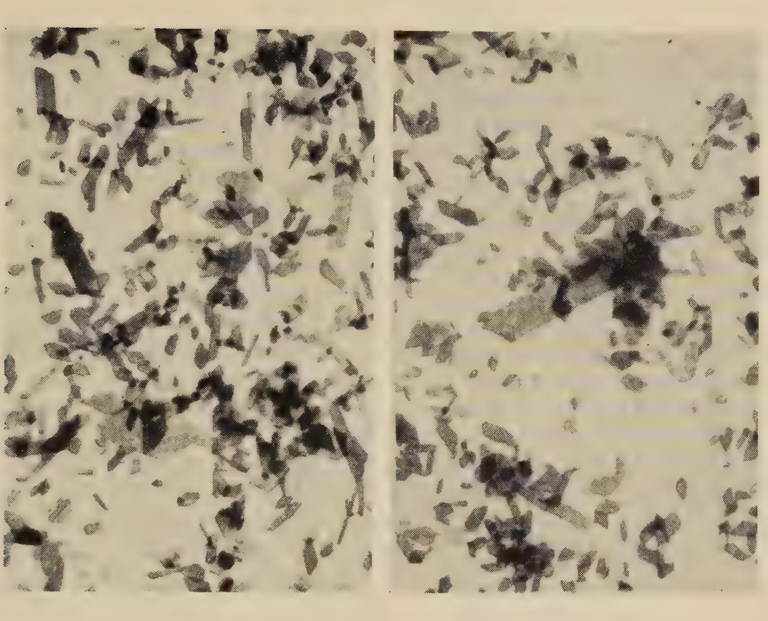

Fig. 1. Isolated chromoplasts from carrots (×1000).

especially submicroscopic fragments of the chromoplasts ("chromoplastic matter"), however, have a different composition from that of whole chromoplasts, and they have to be removed if the composition of whole chromoplasts is to be analysed.

For the reasons mentioned above, highly purified chromoplasts could be prepared only with small yields (5–10 mgs from 1.5 l of carrot juice). The preliminary analysis reported here was therefore limited to the attempt to obtain, by micro-analytical and cytochemical methods, a general idea of the composition of the chromoplasts and to test for some biologically important substances.

ANALYTICAL METHODS

"*Total carotene.*" 0.3–0.8 mgs of dried chromoplasts were extracted with petroleum ether until the residue was colorless; the extracts were collected in a volumetric flask and the color measured with the Evelyn photoelectric colorimeter (filter 440). The standard curve was made with crystalline carotene from carrots recrystallised twice from petroleum ether. The pigment extract consisting mostly of α- and β-carotene has not been separated into components chromatographically for this preliminary analysis.

"*Total lipids.*" 10–20 mgs of dried chromoplasts were extracted in a test tube with anhydrous ether. The ether extract was separated by centrifugation, and the extraction of the residue by ether continued 3–4 times until the ether was colorless; the extraction was continued three times with alcohol–ether (1:1), one time with alcohol, and the residue of the collected extracts was weighed.

Total N and protein N. N was determined by the micro-Kjeldahl method. The determination of protein N was carried out by extracting the dried chromoplasts successively with dry ether, ether-alcohol (1:1), alcohol and cold TCA (5 per cent) and determining N in the residue by the micro-Kjeldahl method after digestion with H_2SO_4.

Total P and P fractionation. P was determined by the method of Fiske and Subbarow (5) after digestion of the samples with H_2SO_4 and HNO_3. P was fractionated according to Schneider (16) by treating the chromoplast suspension successively with cold TCA (5 per cent) for the extraction of acid soluble material, with alcohol, ether and ether-alcohol (1:1) for the extraction of lipids and with TCA (5 per cent) at 90° C for the extraction of RNA. In other experiments, RNA-P was determined according to Schmidt and Thannhauser (15).

Nucleic acid. Nucleic acid was determined spectrophotometrically according to a slight modification of the method of Ogur and Rosen (12). The chromoplast suspension was treated with 0.01 N $HClO_4$, the sediment extracted successively with alcohol once, with ether 3–4 times, with alcohol-ether (1:1) twice, with alcohol once, with cold 0.2 M $HClO_4$ twice. The nucleic acid was then extracted by heating with 1 N $HClO_4$ for 30 min. at 90° C and the absorption at 260 mμ of the $HClO_4$ extract was measured in the Beckmann UV spectrophotometer. Pure yeast nucleic acid (Merck) in 1 N $HClO_4$ (heated for 30 min. at 90° C) was used as standard and its P content as reference (12).

Ether soluble Ca. The residue of the "total lipid" extract obtained as indicated above was dry ashed in a platinum crucible, the ash dissolved in hot 0.1 N HCl, the solution transferred to a micro-centrifuge tube, the acidity decreased with sat. sodium acetate and the Ca precipitated with ammonium oxalate. After standing over night, the Ca-oxalate was centrifuged off, washed with diluted ammonium oxalate on the centrifuge, dried and transformed into $CaCO_3$ by heating for one hour at 500° C in a muffle oven. The $CaCO_3$ was titrated with N/100 HCl with a micro-burette (9).

Ash. The chromoplasts were dry ashed in a platinum crucible and the ash weighed on a micro-analytical balance.

Fe. The ash was dissolved in 0.1 N HCl and the absorption of the pigment developed with o-phenanthroline was measured in the Beckmann spectrophotometer at 508 mμ (9).

RESULTS OF PRELIMINARY CHEMICAL ANALYSIS

The results of the chemical analysis are summarised in Table I. The first column refers to percentage of components per dry chromoplasts. The second column indicates percentage of components in the "stroma" (chromoplasts minus carotene), and is calculated from the figures of the first column and the "total carotene" content of the samples.

TABLE I

Preliminary analysis of isolated chromoplasts of carrots.

	Per cent of dry chromoplasts	Per cent of "stroma" (chromoplasts minus carotene)	Number of experiments
1. Total carotene	20–56	–	32
2. Total lipids (average)	72	58	3
3. Total N	2.2–3.2	3.8	9
4. Protein N (average)	2.5	3.5	4
Proteins (protein N × 6.25) .	15.6	21.9	
5. Total P	0.6–1.0	1.16	19
6. Acid soluble P	0.1–0.18	0.16	5
7. Lipid-P	0.4–0.65	0.83	5
Phospholipids (Lipid-P × 25)	10.0–16.4	20.7	
8. RNA-P	0.07–0.1	0.13	5
9. Nucleic acid (U.V.-spectrophotometry)	0.53	0.72	2
10. Total ash	4.5	6.2	2
12. Ether soluble Ca	0.31	0.42	2
12. Fe	0.03	0.04	2

From the data of Table I the composition of the chromoplasts of carrots can be estimated. The carotene content of the chromoplasts varies from 20–56 per cent. (The variation of the carotene content will be discussed in detail below.) Chromoplasts, with an average carotene content of about 30 per cent, contain, in addition to the pigments, 30–40 per cent colorless lipids, 15 per cent proteins and 4.5 per cent ash. The phospholipid content as estimated from the lipid-P amounts to 15 per cent of the whole chromoplasts, or 21 per cent of the stroma, or about 40 per cent of all colorless lipids. If the ether soluble Ca is derived from the Ca salt of phosphatidic acid as will be discussed below, this substance would represent about $^1/_3$ of the phospholipids of the chromoplasts (7 per cent of the stroma). The ribonucleic acid content of the protein fraction as calculated from the ultraviolet absorption at 260 mμ and the protein N amounts to 3.3 per cent. The figure would be considerably higher if the RNA-P determinations were taken as basis of calculation. This discrepancy has to be clarified later when the P containing organic compounds of the chromoplasts have been analysed in greater detail.

CYTOCHEMICAL TESTS WITH ISOLATED CHROMOPLASTS OF CARROTS

In the following paragraphs, procedures will be described for testing the purity of isolated chromoplasts microscopically, and for preserving the stroma and the stroma proteins for cytochemical reactions. Cytochemical tests for ribonucleic acid and desoxyribonucleic acid were applied.

During the experiments described below, apparently intact "stromata" were seen quite often. They appeared as strongly reflective, thin rectangular plates with the characteristic "border fibers" (21), and were free of pigments. The interesting feature of these stromata was their homogeneous aspect. If this was not an artefact caused by the reagents, it would indicate that the fibrillar or lamellar structure demonstrated formerly (21) has submicroscopic dimensions. Electron microscopy would thus be the appropriate method for the morphological study of the stroma. The procedures for the preservation of the stroma, described below, may facilitate electron microscopic studies. Since the pigment content and thus the proportion of pigments to stroma varies greatly in the chromoplasts of carrots, chromoplasts with relatively low carotene content (20–25 per cent) are more favorable for the study of the stroma than those with higher carotene content.

Estimation of purity of isolated chromoplasts

Simple staining with basic dyes such as pyronine or methylene blue allows the immediate recognition of contamination by submicroscopic cytoplasmic granules or by disintegration products of the chromoplasts themselves. For the contaminating material, probably due to its ribonucleic acid content, is intensely stained by basic dyes while the chromoplasts remain mostly unstained except at their envelopes ("border fibers") where the stroma is accessible to the dye (21). Highly purified chromoplast preparations thus are not stained by methylene blue (except the "border fibers") while impure or disintegrated preparations, stained by methylene blue, show dark blue clumps of agglutinated submicroscopic granules, and a blue rim of these granules concentrated at the edge of the preparation on the microscopic slide.

Preservation of the stroma and of the stroma proteins for cytochemical tests

As was mentioned in the introduction, the "dissolution" of the chromoplasts of carrots after treatment with organic solvents has led many investigators to regard them as crystals of carotene. According to the above analysis, approximately $^3/_4$ of the chromoplasts are soluble in ether. It is thus plausible that treatment with lipid solvents will cause disintegration of the chromoplasts. At least partial preservation of the stroma is possible, however, by procedures which preserve the proteins and a part of the lipids while dissolving or oxidizing most of the pigments. Methods of this kind, used before, were treatment with weak iodine solution (20), treatment with hot water, or long enclosure on a microscopic slide with resultant fading of the pigments (21). The following methods for the preservation of the stroma are probably superior to those mentioned since they are less drastic and can be executed quickly.

Treatment with 70 per cent acetone. It has previously been indicated (20) that treatment with 70 per cent acetone will cause a part of the chromoplasts to fade and will make their stroma visible while often causing recrystallisation of the dissolved pigments on other chromoplasts. The phospholipids of the stroma are insoluble in acetone; this explains why acetone preserves the stroma better than other lipid solvents. The solvent was applied either by immersing a microscopic slide with the dried and heat fixed chromoplasts into 70 per cent acetone, or by adding acetone to an aqueous chromoplast suspension to make up to 70 per cent. After standing $^1/_4$–1 hour, a drop of the suspension was dried on a microscopic slide.

Treatment with n-butanol. By shaking an aqueous chromoplast suspension with n-butanol, the chromoplasts will lose most of their pigments to the butanol, and the faded chromoplasts can be separated from the aqueous layer by centrifugation. A part of the chromoplasts is concentrated in a thin layer at the butanol-water interface and can be adsorbed to a glass rod and transferred to a microscopic slide for cytochemical examination. After treatment with n-butanol there will be found, besides chromoplasts which have lost part or all of their pigments, others which have become more intensely colored by secondary crystallisation of the dissolved pigments on them (22).

The use of n-butanol was recommended by Morton (11) for the purpose of bringing enzymes into solution from their insoluble complexes with lipids. The above experiments indicate that n-butanol is also favorable for the separation of the proteins and lipids of chromoplasts.

Treatment with ether. By shaking an aqueous chromoplast suspension repeatedly (3–5 times) with ether in a separatory funnel (and discarding the ether layers with the extracted pigments), the chromoplasts in the aqueous layer are broken into fragments with irregular outlines which have lost most of their pigments but appear to have retained the proteins and part of the lipids. Since structure seems to be partially preserved in these fragments, they are favorable for the localisation of cytochemical reactions in the stroma, especially the stroma proteins. The fragmentation by ether was followed microscopically and was interrupted when the desired degree of fragmentation and decolorisation was reached. The fragments remained in stable suspension in the aqueous layer and were not agglutinated during the treatment.

In order to free the proteins of the stroma from all pigments and most of the lipids, ether was applied in another way. Microscopic slides on which a highly purified chromoplast suspension had been dried and fixed by heat, were rinsed with or briefly immersed in ether. After this treatment, the slide contained masses of finely granular material and the structure of the chromoplasts appeared, at first sight, completely destroyed. Careful observation showed, however, that dispersed between the granular material many extremely fine rectangular or rod-shaped stromata or rests of stromata were still recognizable.

In order to obtain the proteins of the stroma completely free from lipids, the slides with the chromoplasts (dried in and fixed by heat) were rinsed with ether as above, and then treated with hot alcohol and alcohol-ether (1:1) in a beaker. After another fixation over the flame, they were used for cytochemical tests.

Cytochemical tests for ribonucleic acid and desoxyribonucleic acid

According to Brachet (2), ribonucleic acid can be recognized histochemically by staining with pyronine. No staining will take place in control slides treated with ribonuclease or hot trichloracetic acid. The test was applied to chromoplast suspensions treated with ether as indicated above. A highly purified chromoplast suspension which contained no pyronine or methylene blue stainable granules, was shaken 3 4 times with ether in a small separatory funnel and the ether layers with the extracted pigments discarded. A few drops of the weakly yellow "stroma suspension" was dried on a microscopic slide and fixed by heat. Another slide was prepared by drying a few drops of the chromoplast suspension on it, fixing by heat and then briefly immersing the slide in ether. After these treatments, both slides were strongly stained by pyronine. The stained material on the first slide consisted of small fragments (1–3 μ diam.) of the stroma with irregular outlines, and on the second slide of clumps of submicroscopic granules. When slides with the same ether-treated chromoplasts were incubated for 1 hour in ribonuclease solution at 56° C or heated in 5 per cent TCA for 20 minutes, the stroma fragments or clumps of submicroscopic granules were not stained by pyronine while control slides kept for 1 hour in dist. water at 56° C were still basophilic. These experiments indicate the ribonucleic acid content of the stroma.

For the examination of DNA, slides with isolated chromoplasts were treated with ether, ether-alcohol (1:1) and hot alcohol as indicated above, hydrolysed for 10 min. in 1 N HCl at 60° C and then treated by the Feulgen stain. Staining for DNA was negative. (When the lipids were not completely removed, however, the Feulgen stain was strongly positive thus showing high aldehyd content of the lipid fraction.)

HYDROPHILIC PROPERTIES OF CHROMOPLAST SUSPENSIONS

The most surprising property of isolated chromoplasts in aqueous suspension is the hydrophilic character of particles containing approximately 70 per cent lipids. After sedimentation in the centrifuge, highly purified chromoplasts go into aqueous suspension with great avidity and remain in stable suspension as long as not too much disintegration takes place. The hydrophilic properties of the chromoplasts appear to exceed greatly those of colloidal carotene of low degree of dispersion (8) though the average particle size of the chromoplasts is many times greater than that of the small carotene crystals in "colloidal" carotene suspensions. It is probable that the surface active (detergent) properties of the phosphatides found above as components of the stroma, contribute to the hydrophilic properties of the chromoplasts.

The hydrophilic properties of isolated chromoplasts are masked in the presence of cytoplasmic granules or granular disintegration products of the chromoplasts themselves. These granules tend to be adsorbed to the chromoplasts causing agglutination of the chromoplasts and granules. Since tendency towards disintegration of isolated chromoplasts increases with decreasing carotene content, chromoplasts with higher carotene content (30–50 per cent) remain longer in stable aqueous suspensions (for days or weeks) than do chromoplasts with lower carotene content (20–30 per cent).

Brief boiling does not affect the "colloidal" stability of isolated chromoplasts in suspension, and no agglutination takes place. Congelation of the chromoplast suspension for only a short time, however, causes agglutination (denaturation) of the chromoplasts and loss of "colloidal" stability. After dessication, too, the chromoplasts become completely insoluble (denatured).

Because of the crystalline shapes and optical anisotropy of the pigmented bodies, chromoplast suspensions show very strong "schlieren" formation. Partial or complete agglutination of the bodies as it occurs in the above indicated cases, for example, can be recognised by weakening or cessation of the "schlieren" formation before visible precipitates are formed.

DISCUSSION

According to the above analysis, the carotene content of isolated chromoplasts varies from 20 to 56 per cent. The question arises whether these variations are caused by contamination or disintegration of the chromoplasts or by oxidation of the pigments during isolation. It is quite probable, however, that these factors are not responsible for the variations of the carotene content. For the degree of purity and disintegration of the chromoplasts could be well judged by staining with methylene blue, and variations of the carotene content were also found in preparations which appeared microscopically pure and not disintegrated. Loss of pigments through oxidation probably did not play a significant role in the variations of the carotene content since control experiments showed a relatively great stability of the carotene in chromoplast suspensions kept at low temperature. This stability of the carotene in the chromoplasts is probably due to accompanying phospholipids; for lecithin had been formerly observed to inhibit the autoxidation of carotene in colloidal solutions (8).

As indicated by Hansen (7) and other investigators, the carotene content of carrots varies with age, temperature during growth and the variety of carrots. These same factors probably are also responsible for the differing carotene content found among isolated chromoplasts. The lowest carotene values (20 to 25 per cent) of isolated chromoplasts were found in summer, and the highest (40 to 56 per cent) during autumn and winter. The same seasonal variations were described for the carotene content of whole carrots (7), which increased from summer to winter in the same proportion (2 to 3 times) (7) as it was found for isolated chromoplasts. This seems to indicate that the chromoplasts synthesize different amounts of pigments in relation to age, variety and temperature during growth while the amount of chromoplastic matter remains approximately the same in the age group of carrots examined (estimated age 3 to 9 months). It will be interesting to elucidate whether the low carotene content of very young carrots is due to the smaller proportion of chromoplasts in the carrots or to the lower carotene content of the chromoplasts. This can be determined by measuring the carotene content of the whole carrots and of the chromoplasts of the same carrots since the concentration of carotene in whole carrots divided by the concentration of carotene in the chromoplasts is equal to the proportion of chromoplasts in the carrots.

The further analysis of the lipid fraction of the chromoplasts will be of especial interest since the biological precursors of the carotene may be expected to be among them. According to the above analysis, about 40 per cent of the colorless lipid fraction consists of phospholipids but the rest has not yet been examined. The ether soluble Ca of this fraction may be derived from the Ca-salt of phosphatitic acid. This substance has been shown by Chibnal and Channon (4) to occur in green leaves where it is probably a component of the chloroplasts (14), and its probable presence in carrots has been indicated by Hanahan and Chaikoff (6). The possible association of phosphatidic acid with lecithin in the chromoplasts of carrots calls to mind recent observations by Kornberg and Pricer (10) that phosphatidic acid may be the biological precursor of lecithin.

The above analysis has shown that carotene is associated with nucleoproteins in the chromoplasts of carrots. This was concluded from RNA-P determinations, ultraviolet spectrophotometry and cytochemical tests. The association of the carotinoid pigments with nucleoproteins in carrots was also noted by Chargaff (3) and in tomatoes by Bracco and v. Euler (1) though the fractions examined by these investigators probably consisted of mixtures of chromoplasts (or chromoplastic matter) and cytoplasmic matter. Perhaps the biological role of the carotinoid pigments is related to their association with nucleoproteins which are now generally considered to function in protein synthesis and genetic processes. Whether the chromoplasts of carrots contain, in addition to RNA, small amounts of DNA can be tested only when larger quantities of chromoplasts have been isolated. The Feulgen test for DNA was negative as indicated above.

The nucleoprotein content of the stroma seems to support the interpretations of the writer concerning the development of the fibrillar, spiral and polygonal shapes of the chromoplasts from submicroscopic cytoplasmic precursors (22). For association of nucleoproteins with phospholipids is known to occur in submicroscopic cytoplasmic fractions (''microsomes'') separated from cells by several investigators, and the concentration of ribonucleic acid and phospholipids in this submicroscopic particulate matter is of the same order of magnitude as found above for the stroma of the carrot chromoplasts. Electronmicroscopic studies of animal cells by Palade (13), Sjöstrand (17) and Sjöstrand and Rhodin (18) have revealed submicroscopic lamellae (membranes) in the cytoplasm, mitochondria and in nuclear membranes, supposed to consist of alternating layers of proteins and lipids (18). They seem to be analogous to the submicroscopic fibrils or lamellae of chromoplasts and chloroplasts (22).

SUMMARY

The carotene content of the chromoplasts of carrots varies from 20 to 56 per cent. The pigment-free portion ("stroma") contains approximately 58 per cent ether and alcohol soluble matter, 22 per cent proteins and 6 per cent ash. 0.83 per cent lipid-P, corresponding to 21 per cent phospholipids, 0.4 per cent ether soluble Ca and 0.04 per cent Fe were found in the stroma. The protein fraction of the stroma contains approximately 3.3 per cent ribonucleic acid.

Procedures were described to remove the pigments and to preserve the stroma for histochemical tests. Cytochemical tests for RNA were positive and for DNA negative.

The strongly hydrophilic properties of the chromoplasts in aqueous suspensions were attributed to the high phospholipid content of the stroma.

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FERTILIZATION AND ACTIVATION OF SEA URCHIN EGGS IN GLASS CAPILLARIES

I. MEMBRANE ELEVATION AND NUCLEAR MOVEMENTS IN TOTALLY AND PARTIALLY FERTILIZED EGGS

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Elevation of the fertilization membrane in the egg of the sea urchin has been studied under a number of different experimental conditions. Recently Hörstadius and Runnström (2) found that eggs fertilized in glass capillaries exhibited elevated fertilization membranes on only a part of the egg surface not in contact with the glass. Most often this membrane was not elevated on the side of sperm penetration, and contraction of the cytoplasm, which in spherical eggs takes place around the site of sperm entrance, often occurred instead on the opposite side where the membrane arose. Notable variation was encountered in the results, but it was concluded that stretching of the surface layers of the egg played an important role in this local inhibition of membrane elevation.

The experiments of Hörstadius and Runnström were repeated as a preliminary to employing the capillary fertilization method for other problems; the results of these authors were essentially confirmed, except that possibly even more variation was encountered. Other techniques were attempted in order to reduce this variation. As a consequence it was learned that the results depended largely upon whether the eggs were held stationary or allowed to move during insemination.

A less studied aspect of fertilization is the behavior of the sperm and egg nuclei in preparation for syngamy. The capillary fertilization method is useful to study the swelling, migration and fusion of the nuclei, for the position of the egg nucleus can be altered with respect to the point of sperm entrance by the manner in which the egg is oriented when drawn into the capillary.

In experiments on eggs elongated to over 150 microns, a number of cases were observed in which the fertilization impulse failed to pass over the entire egg surface. In such partially fertilized eggs, the egg nucleus was occasionally located in unfertilized cytoplasm, so that its subsequent behavior varied from the normal pattern. The results presented here on nuclear phenomena are preliminary and based on observation of less than fifty eggs, but these observations, although limited in number, are presented in hope that they will prove useful later in interpreting some of the factors involved in the behavior of the nuclei preparatory to syngamy. Discussion of the literature is postponed until a subsequent paper which will deal with the problem of nuclear movements in more detail.

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METHODS

Eggs from both the littoral and deep water forms of the sea urchin *Psammechinus miliaris* were obtained from animals collected in the region around the Kristineberg Zoological Station on the Swedish west coast. The jelly layer was largely removed by 10–15 seconds exposure to acid sea water (pH 4.5) followed by flooding with slightly basic sea water (pH 8.4–8.5). Longer treatment with acid sea water resulted in a weakening and increased adhesiveness of the egg surface, often causing the eggs to rupture when drawn into capillaries. It was found that the addition of 0.1–0.2 per cent serum albumin reduced the adhesion of the egg surface to glass without otherwise influencing the results.

The method originally used in the capillary experiments was similar to that described by Hörstadius and Runnström (2) with some modifications. Capillaries of 0.5–1.0 mm diameter were drawn out at one end to a diameter of from 38 to 75 microns. These were fastened into a larger pipette by balls of plasticene (Fig. 1). The pipettes were then worked by mouth under a preparation microscope. Two methods of fertilization were employed: 1) the eggs were pushed down to the tip of the pipette to meet a freshly diluted sperm suspension and were then drawn up again for observation with the preparation microscope, and 2) the capillary containing the unfertilized egg was sealed at both ends on a slide by drops of ordinary immersion oil (Fig. 1); sperm were introduced through the oil seal by a micropipette.

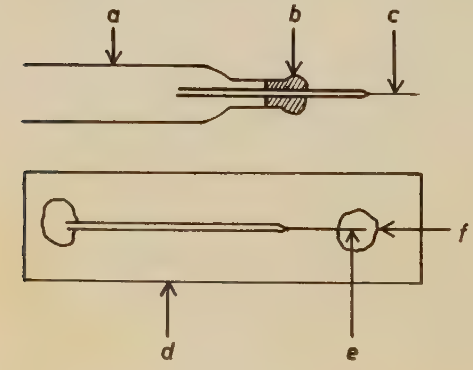

Figure 1. Capillary fertilization method: (a) pipette, (b) plasticene, (c) capillary, (d) glass slide, (e) position of the egg, and (f) a pool of immersion oil.

With this latter method the eggs were kept immobile during insemination; this result was also obtained by drawing sperm into the pipette at a considerable distance from the egg before sealing the capillary in oil, so that the sperm-egg contacts took place after the egg became fixed in position. Having the eggs in thin-walled capillaries under oil provided an exceptionally good opportunity for observation of cortical changes, nuclear migration and other changes associated with fertilization. Experiments were carried out at room temperature (18–22 degrees C.).

The type of glass from which the capillaries were made had an influence upon the results. Best membranes arose in capillaries made from soda glass; pyrex and Jena "normal" glass were easier to use and the eggs had less tendency to adhesion, but membranes were always poor or lacking. Confinement in any of the capillaries apparently did not have deleterious effects on the eggs; often they appeared quite normal after periods as long as 12 hours in the capillaries.

RESULTS

Membrane elevation in slightly elongated eggs. Employing the technique of Hörstadius and Runnström (2), the results of these workers were essentially confirmed: membrane elevation is often inhibited on one side of an elongated egg, and frequently on the side of sperm penetration. Cytoplasmic contraction on the side of membrane elevation was also observed in a number of cases. However, when the eggs were sealed under oil prior to insemination and thus prevented from moving against the glass surface during sperm attachment, normal membranes arose on the surfaces of the eggs not in actual contact with the glass, and no contraction was observed (Fig. 2). Furthermore, the membranes did form on the surface in contact with the glass, for eggs held vertically in their capillaries slid back and forth in their membranes under the influence of gravity. No evidence of granulation of the membranes was detected.

As far as could be determined, partial membrane formation in slightly elongated eggs as first described by Hörstadius and Runnström (2) results from movement of the egg surface against the glass surface *during sperm attachment or penetration.* Considerable previous movement of the egg in the capillary does not hinder normal membrane elevation if the egg is subsequently held stationary during sperm entry.

Membrane elevation in greatly elongated eggs. When the material was at optimum maturity, it was possible to draw some eggs into capillaries considerably less than 60 microns in diameter. In this way, eggs from 150-410 microns in length were obtained (Fig. 3). Fertilization of such elongated eggs in capillaries usually resulted in membrane elevation only on the side of sperm penetration. Closer observation showed clearly that the cortical

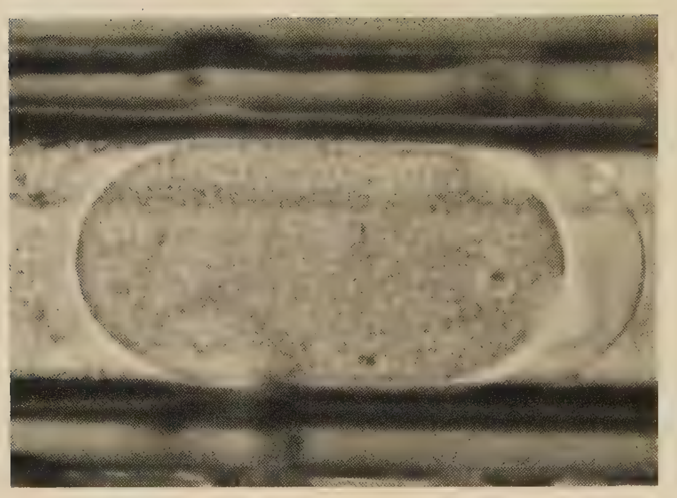

Figure 2. *Psammechinus* egg fertilized in a capillary, showing the fertilization membranes on both free ends.

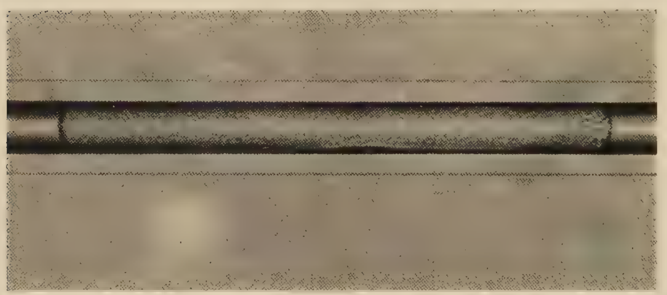

Fig. 3. An egg elongated to 410 microns in a capillary of small bore.

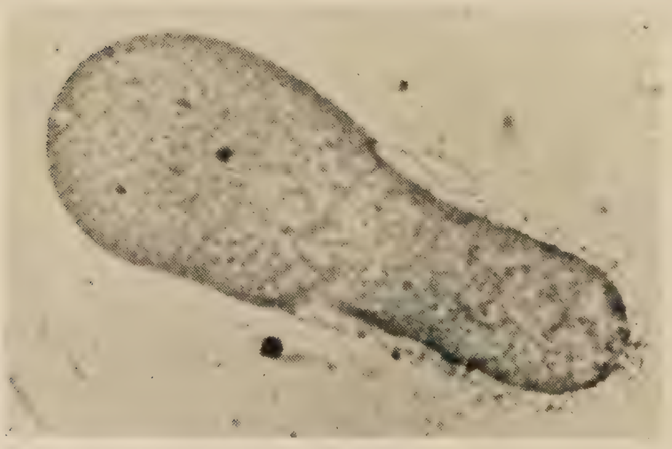

Figure 4. A partially fertilized egg treated with hypertonic sea water after removal from its capillary.

granule breakdown had failed to pass over the entire egg surface, thus leaving part of the cortex fertilized and the remainder unfertilized. That this was in fact true was demonstrated by: 1) continued complete fertilizability of the entire unfertilized part, 2) characteristic dark-field colors for the two parts, 3) formation of a hyaline layer on the fertilized part, 4) different behavior of the two parts in hypertonic sea water (Fig. 4), and 5) contraction of the fertilized part away from the walls of the capillary, resulting in an elongation of the fertilized part. When such half-fertilized eggs are removed from their capillaries, the fertilized portions always maintain their cylindrical shape, whereas the unfertilized parts become almost spherical.

The egg nucleus: its position and migration. Unfertilized eggs were drawn into capillaries of small diameter (40–60 microns); the behavior of their nuclei indicated that the egg nucleus is normally fixed in relation to a portion of the surface in the region of the animal pole. When a portion of the egg surface 20–40° from the animal pole was seized by the end of the capillary, and the egg drawn in and held stationary under an oil seal, the nucleus was observed to move as much as 15–20 microns in only a few minutes. When the animal pole surface directly preceded the egg into the capillary, there was little or no subsequent nuclear movement. This strongly indicated the presence of a mechanism (possibly fibrillar) to maintain the nucleus in a relatively fixed position with respect to the animal pole surface prior to fertilization.

In normal spherical eggs, one of the first indications of complete activation is the swelling of the egg nucleus. When eggs assume cylindrical form in capillaries, this swelling becomes an elongation in the plane of the long axis of the egg; the nucleus begins to bulge in the direction of the point of sperm entrance after about seven minutes. This nuclear elongation takes place whether the egg nucleus is located in fertilized or unfertilized cytoplasm, although in a few of the latter cases merely spherical swelling occurred. Often mechanical injury at one part of the surface also caused the nucleus to elongate just as if the point of injury had been the site of sperm entrance. Eggs which were activated by periodate treatment of one free surface in capillary tubes also exhibited nuclear elongation in the plane of the long-axis of the egg. When the egg nucleus was very close to the point of sperm entrance, its elongation was often toward the sperm, and not in the long-axis of the egg.

In spherical eggs the egg nucleus migrates approximately 20–30 microns to the center of the cell, where it fuses with the sperm nucleus, forming the synkarion. In elongated cylindrical eggs, the distance between the egg nuclei and the point of sperm entrance varied from 65–170 microns, but in all cases where the egg nuclei lay in fertilized cytoplasm, it was possible for them to migrate in the direction of the point of sperm entrance. The longest migration observed was 70 microns in 38 minutes. The fastest migration was 35 microns in 10 minutes (Fig. 5).

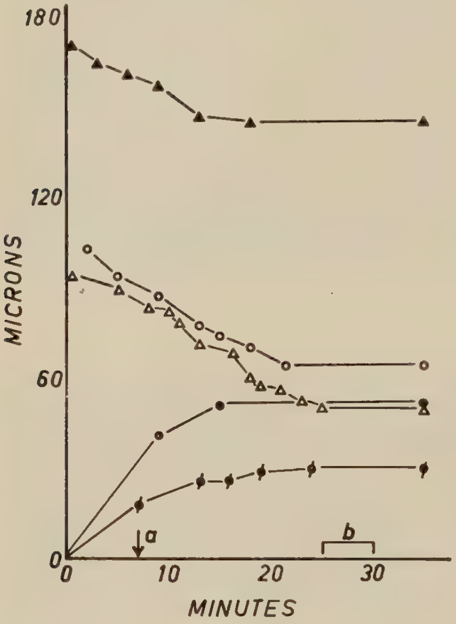

Fig. 5. Migration of the egg nucleus toward, and the sperm nucleus away from, the point of sperm entrance (0 on the ordinate). (a) is the approximate time of elongation of the egg nucleus, and (b) is the approximate time of disappearance of the sperm aster. (•) represents the migration of a sperm nucleus in a capillary of large diameter, and (∅) in a capillary of small diameter. Each curve represents a different experiment.

If the egg nucleus does not originally lie in the axis of the cylindrical center of the egg, it comes to occupy this position soon after activation and before it begins to migrate.

The egg nucleus is generally unable to migrate if located in unfertilized cytoplasm. However, egg nuclei surrounded by unfertilized cytoplasm frequently swell spherically, or sometimes elongate, and a few cases of nuclear breakdown in unfertilized cytoplasm were observed. The latter apparently occurred as a result of similar changes taking place in the fertilized cytoplasm associated with the breakdown of the membrane of the sperm nucleus.

The penetration and migration of the sperm nucleus. The sperm nucleus penetrates at a rather constant rate to a position which is largely determined by the diameter of the capillary. In small-bore capillaries (40–55 microns in diameter), this migration stops after 20–30 microns, whereas in larger bore capillaries it may continue up to 45–50 microns. The migration of the sperm nucleus is coincident with the growth of its aster (cf. (1); the cessation of migration is also coincident with the disappearance of the sperm aster. Since in small-bore capillaries the diameter of the aster approaches that of the capillary, it seems plausible that the cortex may interfere mechanically with the movement of the aster.

The importance and possible role of the sperm aster in nuclear movements were indicated by the following two observations. 1. In one instance, a sperm entered an elongated egg whose cortex had been injured so that the cortical granules were missing on part of the surface; this sperm did not form an aster, and neither of the nuclei migrated. 2. In another egg, two sperms entered, one apparently a little after the first. The second sperm evidently pushed the first ahead of it so that the latter came to lie in a position just twice as distant from the point of sperm entrance as it would have migrated by itself.

In one experiment, an egg was divided in half by a glass needle, and the enucleate half was fertilized in a capillary. The distance that the sperm travelled was identical to that observed in normal eggs, thus demonstrating that the presence of the egg nucleus is not essential for normal sperm penetration path into the egg interior. In this egg, the sperm nucleus became swollen after about 25 minutes as in the normal eggs, and no further changes took place for several hours.

In a few cases where the egg nucleus was immobile in unfertilized cytoplasm, the sperm nucleus continued its migration at a very slow rate until it came in contact with the unfertilized cytoplasm. The sperm nucleus was unable to enter this unfertilized cytoplasm and instead became elongated along the border of fertilized-unfertilized cytoplasm. Here it underwent several abnormal mitotic divisions. It was noted that at the time when the sperm nucleus lost its membrane in preparation for these mitotic divisions, the egg nucleus, located as much as 35 microns away in unfertilized cytoplasm, showed nuclear breakdown but no subsequent spindle formation.

Fusion of the sperm and egg nuclei. Except for a few cases where the egg nucleus was located close to the point of sperm entrance, fusion of the nuclei generally did not occur. Evidently there is a time limit after which the competence for syngamy is lost, even though the nuclei may come to lie within only a few microns of one another.

DISCUSSION

Membrane elevation and partial fertilization. The results obtained in this study make it appear rather probable that the inhibition of membrane elevation on part of the surface of eggs fertilized in capillary tubes is due mostly to movement against a glass surface rather than to stretching, as was believed by Hörstadius and Runnström. In the present study the eggs were often stretched to a much greater extent without inhibition of membrane elevation in areas where the fertilization impulse extended. Partial membrane formation in very much elongated eggs was due to incomplete propagation of cortical granule breakdown and other changes accompanying the fertilization impulse, for this impulse died out along the cylindrical surface of the egg. Thus the coexistence of unfertilized and fertilized cytoplasm in the same egg is confirmed (cf. Runnström and Kriszat (3)).

Nuclear movements and syngamy. From observations around the turn of the century ((4), p. 426) it is clear that the *penetration path* of the sperm is independent of the position or presence of the egg nucleus and due to some reaction between the sperm and the egg cytoplasm, whereas the *copulation path* is influenced by the position of the two nuclei. The observations made in the present study confirm these older ideas and suggest the possibility that the sperm aster (1) may play an important role in the penetration of the sperm nucleus (*penetration path*). The movements which comprise the *copulation path*, however, can be undertaken by either or both of the nuclei. When the movement of either nucleus is hindered, the other is able to migrate. Probably there is movement of both nuclei under normal conditions. It is noteworthy that the egg and sperm nuclei are able to exert some kind of mutual attraction at a distance as great as eight times the distance normally undergone in the movements which comprise the copulation path.

One striking effect of lengthening the copulation path is to delay the meeting of the nuclei and thus prevent their fusion to form the synkarion. The egg has apparently lost its competence for this process soon after it would normally occur in the spherical egg.

The movement of the egg nucleus may be partly accomplished by fibrillar action. A fibrillar system is visible between the approaching nuclei when they are close together. The egg nucleus bulges and otherwise often behaves as if pulled by fibrils.

General cytoplasmic movements (1) may also play a part in the migration of the nuclei; this possibility will be examined later with time-lapse cinematography.

Swelling of the egg nucleus prior to migration may be caused by diffusion of some substance from the activated cytoplasm, as evidenced by the fact that such swelling takes place even in unfertilized cytoplasm and can be caused by artificial activation or local injury as well as by the spermatozoan.

Breakdown of the egg nuclear membrane may also be caused by a diffusing substance from the fertilized cytoplasm; however, the contents of the egg nucleus are not able to react with the unfertilized cytoplasm to form a mitotic spindle.

As this subject will be taken up in greater detail in a subsequent paper, a discussion of the literature on the subject is postponed until that time.

SUMMARY

Psammechinus miliaris eggs were fertilized in glass capillaries of small diameter, the ends of which were sealed with drops of oil. Complete membrane elevation occurred on slightly elongated (cylindrical) eggs (110–140 microns) when these eggs were held stationary. Membrane elevation could be partially or totally inhibited by friction of the egg surface against the glass during sperm penetration. In eggs which had been elongated to a greater extent, the fertilization impulse failed to cover the entire egg surface, so that the egg was left only partially fertilized. The unfertilized portions of such eggs remained fertilizable, whereas the fertilized parts underwent the surface and interior changes associated with activation.

Nuclear movements were observed in eggs in various degrees of elongation. The distance over which the egg and sperm nuclei must migrate in order to meet (penetration path plus copulation path) was varied by the manner of orientation of the eggs when drawn into the capillaries. The sperm nucleus penetrated a relatively constant distance from the surface (the penetration path) irrespective of the presence or position of the egg nucleus. These initial movements of the sperm nucleus were coincident with the growth of the sperm aster. Further movements of the two nuclei (copulation path) depended on the positions of the two nuclei and could be undertaken by either or both of the nuclei, which could exert a mutual attraction even when separated by a distance eight times as great as that in the normal spherical egg. The egg nucleus was unable to migrate when surrounded by unfertilized cytoplasm, but swelling and nuclear breakdown could occur. It is therefore believed that these latter phenomena were caused by diffusing substances.

I am very grateful to Professor John Runnström for his helpful advice in planning these experiments and for his active interest in their outcome. I wish to thank Dr. Sven Hörstadius for his careful instructions on the use of the capillary fertilization method, and Dr. Gunnar Gustafson, the director of the Kristineberg Zoological Station, for his kind hospitality during our stay. Sincere thanks are due to my wife, Margaret Allen, for her willing and competent assistance in these experiments.

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II. CORTICAL CHANGES AND THE FERTILIZATION IMPULSE

It has been recognized for three decades that sperm attachment causes a change or impulse to pass over the surface of the sea urchin egg before membrane elevation. Runnström (9) described a wave-like progressive change in the surface under dark-field illumination from a thick, bright yellow to a thinner, brilliant white. Later, Moser (6) noticed another wave: cortical granule breakdown beginning at the point of sperm entry. More recently, Rothschild and Swann (8) have measured the rate of passage of the dark-field color change, but under conditions of illumination which did not show the yellow color observed earlier by Runnström. The relationship of the dark-field color change wave and the cortical granule breakdown wave has not been clarified. Moser believed that the former was indeed caused by the latter; but Rothschild and Swann were of the opinion that the color change was due to the formation, rather than the disappearance of granules. Runnström (9) believed that the dark-field change was due to a change in the orientation of lipid molecules resulting in reduced thickness of the membrane. Recently, Sugiyama (13) has presented evidence from artificial activation studies that cortical granule breakdown occurs as a secondary expression of an invisible fertilization impulse (fertilization wave) which precedes the other changes.

The introduction of the capillary fertilization method by Hörstadius and Runnström (3) has provided an opportunity to examine these surface changes further in order to clarify some of the relationships between the fertilization impulse, the dark-field color change, cortical granule breakdown (or conversion) and membrane elevation. A modification of the method of Hörstadius and Runnström permits even closer observations (1); with this technique it has been possible to obtain a number of partially fertilized eggs (1). The rate and extent of passage of the fertilization impulse has been recorded in 63 such partially fertilized eggs, which have been treated in different ways.

Although the actual amount of information concerning the fertilization impulse is still quite meager, at least three possible mechanisms have already been postulated to account for the phenomena which have been observed. These mechanisms and others are discussed as a possible aid to future experiments.

METHODS

All experiments were carried out at the Kristineberg Zoological Station on the Swedish west coast with eggs of the sea urchin, *Psammechinus miliaris* (both littoral and deep water forms). The jelly layer was removed by 10–15 seconds exposure to acid sea water (pH 4.5), followed by flooding with slightly basic sea water (pH 8.4–8.5). Longer acid treatment made the eggs fragile and adhesive so that they ruptured when drawn into capillaries. The diameter of the eggs varied somewhat but averaged about 97 microns. This figure was used in all calculations for the preparation of Tables I, II and III. Adhesiveness was greatly reduced by the addition of 0.1–0.2 per cent serum albumin to the sea water; this apparently did not otherwise influence the results.

The method of fertilization used has been described in the first paper of this series (1). In most experiments the sperm was introduced through an oil seal into the main capillary by means of a smaller capillary or micropipette. In this way the egg could be observed during sperm attachment and the passage of the fertilization impulse over the egg. Observations of the surface changes were made in both bright- and dark-field illumination.

RESULTS

Insemination and sperm attachment. Sperm were introduced at some distance from the egg so that the preparation could be watched under the oil immersion objective even before sperm attachment had taken place. It was noted repeatedly that it was rarely one of the first ten sperms to come in contact with the egg that succeeded in penetrating. Furthermore, very often the eggs failed to be fertilized even though 10–20 sperms were in contact with the egg surface. Often a second addition of sperm resulted in fertilization. The sperm usually entered fairly near the center of the area of the egg surface exposed to the sea water. Although sperm sometimes were able to pass between the egg surface and the glass, fertilization on the distal side of the egg occurred with this method in only two cases during the season. Sperm penetration never occurred on the surface in contact with the glass.

The first indication of successful sperm attachment was a roughening of the cortex as seen in tangential view. This roughening was evidently caused by the breakdown of the cortical granules. Shortly afterward the fertilization membrane elevated on the proximal end of the egg in a normal manner, with a latent period before the membrane arose at the distal end. It could be seen that the membrane appeared on the distal end after the wave of cortical granule breakdown had passed over the entire surface.

Qualitative aspects of the fertilization impulse. The breakdown of the cortical granules was followed in greater detail under oil immersion (Zeiss objective: 60 ×, N. A. = 0.7). As mentioned above, the beginning of this cortical granule breakdown was detected by a roughening of the surface in tangential view. The top surface of the egg was brought immediately into focus so that the individual granules could be observed. The granules did not break down in a moving border as Moser (6) described in *Arbacia*. Instead, at any time there was a region 20–30 microns wide in which the granules were breaking down seemingly at random (cf. Endo (2)). This region of random granule breakdown moved along the egg surface at a decreasing rate of speed. Each granule could be seen to begin brownian movement immediately before it rather explosively disappeared. No swelling of the granules was observed. In eggs in which the fertilization impulse passed over the entire surface, an increasing rate of passage was detected at the distal end. When the fertilization impulse did not extend over the entire egg, it died out at some point on the cylindrical surface (Fig. 1). It has been mentioned previously (1) that while the portion of the surface with cortical granules intact (the *unfertilized portion*) retains its unfertilized character and remains receptive to sperm, the part with its cortical granules completely broken down forms a fertilization membrane and later a hyaline layer and is not receptive to sperm (the *fertilized portion*). Between these two parts of the partially fertilized egg there is an *intermediate zone* (Fig. 1) in which the cortical granules are smaller than in the unfertilized zone, and become gradually less plentiful toward the fertilized portion.

Parallel observations were carried out under dark-field illumination. The color change from yellow to white follows much the same pattern as cortical granule breakdown. The yellow color fades in a wave-like fashion and is replaced by brilliant white. High power observations on the intermediate zone showed patches of yellow color, presumably caused by the presence of a few cortical granules.

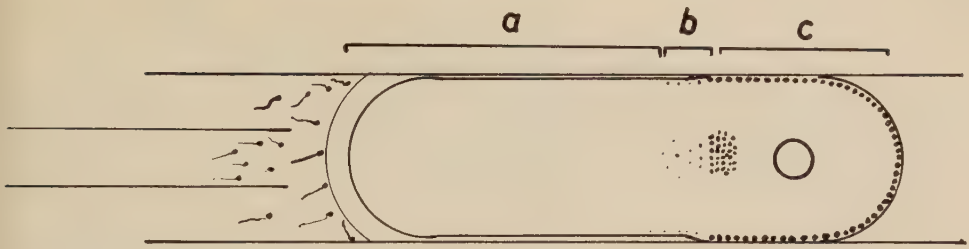

Fig. 1. Diagram of a partially fertilized egg. (a) fertilized portion, (b) intermediate zone and (c) unfertilized portion.

Quantitative aspects of the fertilization impulse. Sixty-three eggs (control and experimentally treated eggs) showing partial fertilization were observed. In these cases the length of the egg and the *impulse linear distance* were recorded. (The impulse linear distance refers to the distance from the end of the egg where the sperm entered to a point on the cylindrical axis under which the cortical granules have retained their normal unfertilized size and distribution.) Since eggs in capillaries assume very nearly the shape of cylinders with half-spheres on the ends, it is simple to calculate the radius[1] of the egg (assuming that the egg volume remains constant) and, from this value, the surface area over which the impulse has passed (Area $= 2\pi r h$, where r is the radius in microns and h the impulse linear distance). The impulse arc distance (the actual surface distance) and the volume of fertilized cytoplasm can also be calculated; however, for reasons to be discussed later, it is believed that these values are of little significance.

The summarized data in terms of impulse surface area are shown in Table I. Where significant differences appear in groups treated differently, the values for per cent difference and probability are given in Table II.

Considered on the basis of impulse area, there appears to be no significant difference between the controls and the eggs pretreated with glycine (0.05 per cent for 10–60 minutes). In both of these groups there does, however, seem to be a striking difference between eggs that are stretched more than 32 per cent and those stretched less than 32 per cent (i. e. per cent increase in surface area). The more stretched eggs showed a smaller percentage of their surface area covered by the fertilization impulse, and, furthermore, a smaller absolute area covered by the fertilization impulse. This inhibitory effect of stretching was even more pronounced in glycine-treated eggs. The degree of stretching is accompanied by and cannot be separated from other

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[1] Ocular micrometer measurements of capillary inside bore were inaccurate due to optical distortion.

TABLE I

Summary of data from incomplete fertilization impulses.

Group	Mean area of impulse (X 104 μ^2)	σ_n of mean	Range	Number of cases
Controls (entire)	2.34	.065	1.63–2.92	31
Controls ($> 32\,\%\,\Delta$ S)[1].	2.15	.087	1.74–2.58	9
Controls ($< 32\,\%\,\Delta$ S)[1].	2.42	.070	2.63–2.92	22
Abnormal eggs[2]	1.26	.140	0.88–1.61	4
Glycine pretreated (entire). . . .	2.26	.080	1.79–2.65	12
Glycine pretreated ($> 32\,\%\,\Delta$ S) .	2.12	.094	1.79–2.43	7
Glycine pretreated ($< 32\,\%\,\Delta$ S) .	2.48	.062	2.24–2.65	5
ATP pretreated	2.66	.160	2.06–3.08	5
Dispermy	2.63	.113	2.46–2.78	2

[1] Eggs that have been stretched more (>) or less (<) than 32 per cent increase in surface area.

[2] Four eggs with abnormally low impulse areas were observed on the same day; these values were different from the controls (cf. Table II).

TABLE II

Comparison of differently treated eggs with respect to the extent of the fertilization impulse.

Groups compared	% difference in area covered by impulse	Probability[1]
Controls: $< 32\,\%\,\Delta$ S/$> 32\,\%\,\Delta$ S[3]	12.5	.022
Controls (entire)/abnormal eggs[2]	90.0	<.01
Controls (entire)/glycine pretreated (entire) .	3.5	–
Glycine: $< 32\,\%\,\Delta$ S/$> 32\,\%\,\Delta$ S[3]	17.2	.002
Controls (entire)/ATP pretreated eggs	14.0	.07

[1] As determined by the *t* test.

[2] Four eggs with abnormally low impulse area values obtained the same day.

[3] Eggs that have been stretched more (>) or less (<) than 32 per cent increase in surface area.

factors, such as increased contact with the glass surface, straightening of the egg surface, and the possibility of contact with toxic substances from the glass.[1]

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[1] However, *Paramecia* and amoebae were able to remain alive for an observation period of 36 hours in capillaries made from the same material and sealed with the same kind of oil.

TABLE III

Complete fertilization impulses in elongated eggs.

Group	Proportion of all eggs with complete fertilization impulses	Proportion of all eggs stretched more than 18.5 %[1] with complete fertilization impulses
Controls	17/48	2/29
Glycine pretreated eggs .	3/15	0/10
ATP pretreated eggs. . .	4/9	2/4
Dispermic eggs	3/5	3/3
Periodate pretreated eggs	2/4	2/2

It can be seen that ATP increases the area of surface covered by the fertilization impulse, and thus must be considered to have a facilitating effect. The entrance of two sperms also causes a significant increase in the impulse surface area. It is especially noteworthy that in the case of the two eggs which showed partial fertilization with dispermy, the entrance of even two sperms did not result in the conversion of the entire volume of cytoplasm to the fertilized condition. In fact, from the standpoint of the diffusing substance theory of activation, as tentatively supported by Rothschild, it is curious that one sperm is not able to completely fertilize an egg whose volume has not been changed by altering its shape.

In all control and glycine-treated partially fertilized eggs, the surface area covered was less than the area that the impulse would have covered in the spherical egg. On the other hand, two ATP pretreated eggs showed impulse areas greater than the area of the spherical egg.

As the degree of stretching of the eggs increased, the likelihood of complete fertilization decreased. Table III shows that complete fertilization occurred less frequently in stretched (more than 18 per cent increase in surface area) control and glycine-treated eggs than in dispermic and ATP or periodate pretreated eggs. This is another way of illustrating the facilitating action of these latter substances on the fertilization impulse.

Rate of propagation of the fertilization impulse. The rate of passage of the fertilization impulse was measured in 10 eggs by noting the time after surface roughening that single granules broke down first at known distances from the point of sperm entrance. These data were recalculated in terms of area covered by the impulse per second, and are compared to the curve obtained for the speed of the dark-field color change impulse by Rothschild and Swann.

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[1] Surface area exceeding $3.5 \times 10^4\ \mu^2$.

(See Fig. 2; only three representative curves are presented here for comparison). Although different curves varied from one another as to rate, slope and form, certain facts became clear. The rate of the fertilization impulse decreased progressively until it died out in the cylindrical portion of greatly elongated eggs. In cases where the impulse passed over the entire surface, its rate increased as it passed over the curved surface at the distal end of the egg, where the area to be influenced by the impulse becomes progressively smaller. There was some indication that the impulse rate in terms of area covered per second was greater in less stretched eggs, although there was too much variability to be sure of this. ATP treated eggs showed a slightly higher rate, but again the amount of variation encountered and the small number of observations make any conclusions only tentative. The fastest area per second rate observed was 0.216×10^4 square microns per second in an ATP pretreated egg. The slowest rate observed that was measureable was 0.003×10^4 square microns per second in a dispermic egg in which the impulse was dying out. Probably the impulse can proceed even more slowly, but under these conditions it is travelling in the intermediate zone, where its effect on the cortical granules is not all-or-none, and so cannot be detected by the present methods.

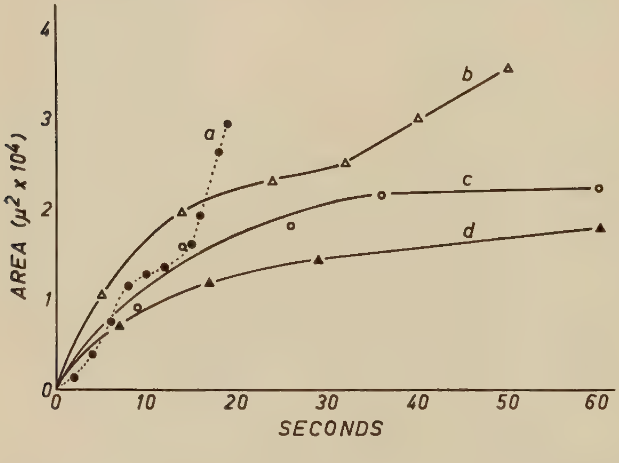

Fig. 2. The rate of the fertilization impulse. (a) a curve for the rate of the dark-field color change impulse, recalculated from data of Rothschild and Swann (8), (b) an ATP pretreated egg (length 188 μ) in which the impulse passed over the entire surface, (c) a dispermic egg (length 423 μ), and (d) a monospermic control egg (length 396 μ). (Curves b, c, and d were constructed from observations on the breakdown of individual cortical granules at different distances from the point of sperm entry.)

DISCUSSION

The random manner of cortical granule breakdown strongly suggests that this process is, as Sugiyama believes, a secondary expression of a submicroscopic change which precedes it. Evidently the fertilization impulse involves a lessening of cortical rigidity, judging from the momentary brownian movement of the granules before breakdown. The appearance of an intermediate zone between the fertilized and unfertilized portions of the partially fertilized egg, with its gradient of shrunken cortical granules leading to the unfertilized part of the egg, suggests that the impulse dies out in a gradual (not all-or-none) manner. The decreased size of the cortical granules in the intermediate zone could conceivably be either an osmotic effect (as these granules do react osmotically by changing their diameter) or an abortive dissolution.

One of the difficulties in studies of surface changes at fertilization is that it is not known for certain how these changes are related. The only fact that seems certain is that the dark-field color change and cortical granule breakdown are related to the extent that they both extend the same distance in partially fertilized eggs. It seems probable, although it has not been proved, that the yellow color is lost when the cortical granules disappear; this was indirectly indicated by observations on the intermediate zone under both bright- and dark-field illumination.

In considering by what mechanism the fertilization impulse could be propagated, the structure of the egg (especially the surface layers) must be kept in mind. It might be assumed, for example, that the impulse could be carried by fibrils in the surface layer or in the cell interior, but such a fibrillar system has not been demonstrated. Furthermore, it would be difficult to explain why an impulse carried on fibrils would decrease, then increase in rate. Similarly, if such a mechanism as diffusion of an activating substance from the sperm through the cortex is postulated, it is first necessary to assume the presence of some sort of membrane or other barrier between the cortex and interior cytoplasm. Even then, it is difficult to explain why an added substance such as ATP or periodate should facilitate a process of pure diffusion, or why, if the cortical volume remains constant, this volume should still not be filled by the amount of diffusing substance introduced by one, or even two, spermatozoans.

For the same reasons, it is doubtful that the fertilization impulse can be explained by diffusion of a substance from the sperm through the interior of the cell; the cell volume is not changed, and yet one, or even two sperm are unable to cause complete fertilization.

There are other mechanisms besides those dealing with diffusion. Runnström and Kriszat (11) have presented evidence that the propagated wave has the properties of a chain reaction which occurs in the cortex. The observation that ATP facilitates the fertilization impulse (both in area covered and in rate) may perhaps support the notion of self-propagation. The increase in impulse rate as it passes over the distal surface of the egg, where the area to be affected is decreasing, is another indication of the chain-reaction character of the phenomenon. That the impulse can proceed at vastly different rates and dies out gradually indicates that it is not an all-or-none phenomenon.

The cortex can be considered to contain evenly spaced *units* of some sort (these *units* could be anything from molecules to submicroscopic granules, but almost certainly not the cortical granules) which can exist in either of two forms: non-activated or activated. The activation of a group of units would then result in these units activating their neighbors; and so on. Thus the role of the sperm would be merely to activate a certain number of units initially, and the impulse would then pass along by a chain reaction of some units converting others to the activated form. The measurements of impulse rate (Fig. 2) show that the number of units activated decreases for each successive unit of time. If the impulse dies out, as it does in the intermediate zone of greatly elongated eggs, it must do so because a number of units considerably less than the total number in that area of the surface have been activated. The character of the intermediate zone with its cortical granule distribution gradient allows the observer to extrapolate in his imagination to these postulated units, whatever they may be. If the units have different thresholds, as the cortical granules evidently also do, it is easy to conceive of how the failure of units to respond would become cumulative, and thus account for the decrease in rate and eventual failure of the impulse. Similarly, the regeneration of the impulse would result from the successful conversion of an ever-increasing proportion of the units as the impulse converges on the distal spherical surface of the egg.

It will be shown in the next paper of this series that periodate treatment alone can initiate a propagating impulse similar in rate and character to that which accompanies fertilization. The effect of periodate (10^{-5}–10^{-4} M) was immediate, in contrast to the other activating agents tried, which took longer times to be effective, and then did not cause an impulse quite like the fertilization impulse. In the present study, subthreshold concentrations of periodate conditioned the egg so that longer (and therefore probably faster) fertilization impulses occurred following subsequent sperm penetration. This facilitating effect of periodate (cf. 4) focuses attention on the carbohydrate inhibitor postulated by Runnström and Kriszat (11); this substance may affect or control the threshold of the postulated units, for apparently periodate can physiologically neutralize this inhibitor.

The action of ATP suggests that high energy phosphate compounds may be utilized in the passage of the impulse; certainly ATP has an improving effect on fertilization (10).

It is even possible that conduction over short distances between the units could be accomplished by the movements of ions or more complex substances; Moser (6) suggested that the release of calcium could pass around the surface of the egg in a self-propagating manner.

It is clear that the egg surface is extremely important for the passage of the fertilization impulse. As the surface is simultaneously stretched, straightened, and brought into contact with a glass surface (any of which could be thought to impede the impulse), the fertilization impulse is gradually slowed down. Evidently the surface or cortical structure is disrupted somewhat as the impulse passes. Stretching or straightening of the surface in the capillaries may already disrupt the structure in which the units we have postulated play an integral part. This may also be indicated by the fact that glycine treatment has apparently no effect on only slightly stretched eggs, whereas it enhances the inhibitory action of further stretching on the passage of the impulse; for it has been shown (14) that glycine, as well as other amino acids, decreases cortical rigidity. The effect of stretching may also be partly due to the fact that the postulated units become further apart. There is some indication that the impulse rate is somewhat less in stretched eggs.

Experiments using the capillary fertilization method are necessarily slow and few in number, but this method does provide unique opportunities for attacking some problems of fertilization.

SUMMARY

Psammechinus miliaris eggs were fertilized in glass capillaries of small diameter, the ends of which had been sealed with drops of oil. As the diameter of the capillaries was decreased, the fertilization impulse (as detected by cortical granule breakdown and the dark-field color change) failed to extend over the entire surface of many of the eggs. In sixty-three cases of such partial fertilization in control and experimentally treated eggs, the area covered by the fertilization impulse was calculated. Stretching, straightening of the egg surface, and contact with the glass: three inseparable variables caused a reduction in the area affected by the impulse. Although glycine had no detectible effect on slightly stretched eggs, it considerably enhanced the inhibition due to greater stretching. Pretreatment of the eggs with ATP or periodate facilitated the passage of the impulse.

The hypothesis of interior diffusion of an activating substance from the

sperm is rejected on the grounds that the cytoplasm of stretched mono- and dispermic eggs was not always entirely converted to the fertilized form although the cell volume was unchanged.

The fertilization impulse is evidently accompanied by a structural change at the surface involving a decrease in cortical rigidity. This impulse is not an all-or-none phenomenon as evidenced by the appearance of an intermediate zone. Rate measurements and other considerations suggest a cortical chain reaction, which can be imagined as initiated by the activation of a certain number of *units* on the egg surface by the sperm. These units when activated are then able to activate others and so propagate the impulse. It is probable that high energy phosphate compounds can be used in the process and that the passage of the impulse is normally opposed by the presence of a carbohydrate inhibitor which can be destroyed by the action of periodate.

I am much indebted to Professor John Runnström for his help and advice in the planning of experiments, and the many discussions of the results. I wish to thank Docent Inga Fischer-Hjalmars for her mathematical analysis of the data, and my wife, Margaret Allen, for her patient assistance.

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III. ARTIFICIAL ACTIVATION AND THE FERTILIZATION IMPULSE

Breakdown of the cortical granules and elevation of the fertilization membrane have been considered as evidence of activation in the sea urchin egg (4). In a recent paper, Sugiyama (6) has distinguished two types of artificial activating agents by their action on eggs, the surfaces of which were only partially exposed. This partial exposure of the surface was accomplished by compressing eggs between a slide and cover slip and changing the solutions bathing them by means of a pipette and blotter. The unexposed surface was observed for breakdown of the cortical granules. With this method, Sugiyama noted that one class of agents caused only local cortical granule breakdown, whereas the other induced a general cortical granule breakdown over the entire egg surface. This latter group of agents was believed to have initiated a true fertilization impulse, whereas the former did not. Another recent study has also suggested that there may exist different pathways of egg activation (1).

The introduction of the capillary fertilization method (3, 2) has made possible further refinements in the technique of partial surface exposure initiated by Sugiyama. In the present study, capillaries were loaded in such a way that the cylindrically shaped eggs were bathed on one side by sea water and on the other by an activating agent. The capillaries were sealed with oil and observed under an oil immersion objective. The eggs of the sea urchin *Psammechinus miliaris* were subjected to such unilateral treatment by activating agents. The types of response observed could be divided into three groups. 1. Mechanical injury (sometimes accidental; otherwise induced by breaking the capillary some distance from the egg), bee venom, and sometimes distilled water and hypertonic sea water, caused local cortical granule breakdown which was restricted to the surface in contact with the activating agent. 2. Urea, and sometimes distilled water and hypertonic sea water, caused local cortical granule breakdown followed by a slow, wave-like granule breakdown of linear rate over the egg surface. This passage did not have the same initial velocity as that observed previously for the fertilization impulse (2). 3. Periodate alone in threshold concentrations (5), or in subthreshold concentrations followed by mechanical injury, caused an impulse of a different character to spread over the egg. The rate of this impulse was indistinguishable from that observed for the fertilization impulse. Complete activation occurred in eggs of the following lengths: 240, 169, 149, 200, 210, 363, 278, and 196 microns. These eggs had been stretched (i. e. their surface areas had been increased by) 7–55 per cent. The likelihood of a normal fertilization impulse passing completely over such stretched surfaces would be extremely small (2).

These results suggest that the fertilization impulse is perhaps more specific than Sugiyama supposed. In compressed eggs it would be very difficult to distinguish between the rate of passage of the true fertilization impulse and a cytolytically induced cortical granule breakdown, for cytolytic change initiated on a band of exposed surface on a compressed egg would presumably be additive with respect to velocity in its progress toward the center of the egg, so that the rate would appear similar to that of the true fertilization impulse. The capillary technique provides the means for distinguishing between cortical granule breakdown from different causes.

It is believed that periodate is the only activating agent of those tried which imitates the action of the sperm in initiating a fertilization impulse in *Psammechinus miliaris* eggs. However, these eggs are not ideal for activation studies, and it is planned to extend these experiments to a more suitable material.

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DIE WIRKUNG VON PURINEN, NUCLEOSIDEN, NUCLEOTIDEN UND ADENOSINETRIPHOSPHAT AUF DIE TEILUNG UND ENTWICKLUNG DES SEEIGELEIS BEI ANWENDUNG VON DINITROPHENOL

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Die Wirkung von nitrierten und chlorierten Phenolen und Kresolen auf die Teilung und den Stoffwechsel des Seeigeleis ist von früheren Autoren eingehend beschrieben worden ((2), siehe auch Zusammenfassung bei Krahl (4)). Nitrophenol hebt die Koppelung auf zwischen den energieerzeugenden Atmungsprozessen und den Reaktionen, welche die freigesetzte Energie in phosphatgebundene Form überführen ((9, 10), siehe auch Lindberg und Ernster (8)). Darum erschien es aussichtsreich zu versuchen, die durch DNP verursachte Hemmung der Zellteilung des Seeigeleis durch Zusatz von ATP wieder aufzuheben. Die früher in dieser Richtung unternommenen Versuche an Seeigeleiern zeigten positive Ergebnisse (5, 6) und sind neuerdings von Barnett (1) bestätigt worden. In einer Reihe von früheren Untersuchungen (5) konnte gezeigt werden, dass ausser ATP auch Muskeladenylsäure sowie Adenosin die Teilungsquote von Eiern von *Psammechinus*, die mit DNP behandelt wurden, erhöhten. Die Wirkung dieser Verbindungen ist nicht einer einheitlichen, einfachen Erklärung zugänglich, da sie nicht unmittelbar auf „energiereiche" Phosphatbindungen zurückgeführt werden kann. Im Zusammenhang mit dieser Fragestellung erschien es aussichtsreich, diese Untersuchungen weiterzuführen und nicht nur die Wirkung von Nucleosiden und Nucleotiden, sondern auch die von Purinen und Pyrophosphat zu prüfen, um zu ermitteln, welchen Einfluss die einzelnen Verbindungsgruppen auf die Teilung und Entwicklung von Seeigeleiern haben, die vorher durch DNP-Behandlung geschädigt worden sind.

MATERIAL UND METHODE

Die vorliegenden Versuche wurden im März mit den Eiern von *Strongylocentrotus droebachiensis* und im April und Mai mit den von *Echinus esculentus* durchgeführt. Alle Versuche erfolgten an der Zoologischen Station Kristineberg an der schwedischen Westküste. Für die Versuche an beiden Arten wurde ein Seewasser von ca. 32–34 ‰ verwendet.

Für alle Versuche wurde ein Raum mit automatisch regulierbarer Temperatur benutzt. Die Temperatur betrug 6–7° C. Für die Versuche mit 2,4-Dinitrophenol kam normales Seewasser zur Anwendung, dem DNP in einer Konzentration von $2{,}5\text{–}5 \cdot 10^{-4}$ M zugesetzt wurde. Das Eimaterial wurde nach der Befruchtung für 45–60 Min. mit einer solchen Lösung behandelt. Danach wurde das DNP durch mehrfaches Waschen mit Seewasser entfernt. Die Eier wurden dann in möglichst gleichen Mengen auf die verschiedenen Seewasserlösungen mit den entsprechenden Zusätzen der zu prüfenden Verbindungen verteilt. Alle Lösungen mit Zusätzen wurden einheitlich auf pH 8,1–8,2 justiert. Das für die Versuche benutzte ATP wurde aus Ba_2 ATP der Sigma Chemical Co. hergestellt (Reinheit ca. 90 %). Das Na_4ATP wurde durch Zusatz von Na_2SO_4 aus dem Ba_2ATP gewonnen, durch Zentrifugieren von dem unlöslichen Rückstand gereinigt und auf 0,1 M gebracht. Die Muskeladenylsäure war gleichfalls ein Präparat der Sigma Chemical Co. Die übrigen Präparate wie Hefeadenylsäure und Hypoxanthin wurden von Hoffmann La Roche, das Guanin von Merck (Darmstadt) und das Adenosin und die Guanylsäure von der Kemikaliebolaget in Stockholm bezogen. Bei den übrigen Chemikalien handelte es sich um reine Laboratoriumspräparate. Die erwähnten Verbindungen wurden durchgehend in drei verschiedenen Konzentrationsstufen, und zwar in den Konz. von 0,004, 0,002 und 0,001 M geprüft. In den übrigen Versuchen kam eine Konz. von 0,004 M zur Anwendung. Für die Versuche wurde nur Material benutzt, bei dem die Befruchtung und die Membranablüftung in quantitativer und qualitativer Hinsicht einwandfrei war. Der Verlauf der Teilung und die Anzahl der geteilten Eier wurde in gleichmässigen Abständen beobachtet und ausgewertet. Der Zustand der Entwicklung wurde über den Zeitraum von mehreren Tagen und in den meisten Fällen bis zur Gastrulation verfolgt. Die zahlenmässige Auswertung der Ergebnisse der Versuche erfolgte durch Feststellung des Teilungszustandes der einzelnen Eier innerhalb der gleichen Fläche. Das Zahlenmaterial wurde für jeden Einzelversuch aus einer Anzahl von 200–300 Eiern gewonnen. Bei den in den Kolonnen der Diagr. 1 und 2 angeführten % Angaben handelt es sich um die Mittelwerte aus je drei Versuchen für jede der beiden angeführten Serien.

ERGEBNISSE

Die Eier von *Strongylocentrotus* wurden 15 Min. nach der Befruchtung für 45 Min. mit $2{,}5 \cdot 10^{-4}$ M DNP behandelt und dann in drei verschiedene Konzentrationen der in Diagr. 1 angeführten Verbindungen gebracht. In Diagr. 1 ist der Teilungszustand der Eier und die Anzahl der Entwicklungsstadien in % nach 24 und nach 48 Stunden dargestellt. Die mit DNP behandelten und dann nur in normales Seewasser überführten Eier zeigen im Vergleich mit den übrigen Versuchen und mit der Kontrolle einen wesentlich schlechteren und geringeren Entwicklungszustand. Bei den mit DNP behandelten und dann in Guanin, Hypoxanthin und Guanosin übertragenen

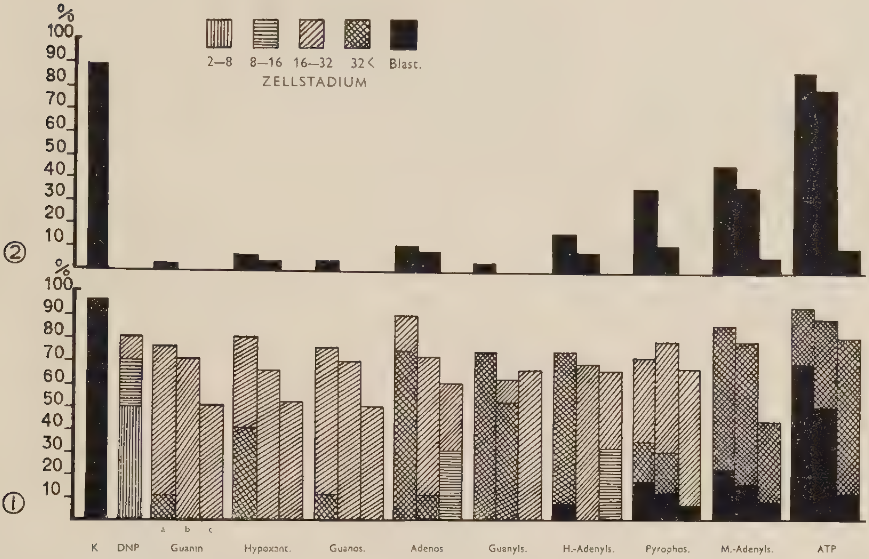


Diagramm 1. Entwicklungszustand der Eier von *Strongylocentrotus*. 15 Min. nach der Befruchtung für 45 Min. mit $2{,}5 \cdot 10^{-4}$ M DNP behandelt. 1. Nach 24 Stunden. 2. Nach 48 Stunden. Konzentration der Lösungen a = 0,004 b = 0,002 c = 0,001 M.

Eiern ist die Teilung etwas weiter fortgeschritten. Bei Adenosin und Guanylsäure zeigt sich eine weitergehende Wirkung. Ein deutlich stärkerer Effekt in bezug auf die Teilung und Entwicklung der Eier ist sowohl quantitativ als auch qualitativ in den Versuchen mit Hefeadenylsäure, Pyrophosphat, Muskeladenylsäure und ATP festzustellen. In diesen Fällen war die Entwicklung nach 24 Stunden teilweise bis zum frühen Blastulastadium fortgeschritten. Die Anzahl der fortgeschrittenen Entwicklungsstadien nimmt in der Reihenfolge von der Hefeadenylsäure bis ATP zu. Ebenso kommt in dem Ergebnis die Wirkung der verschiedenen Konzentrationen zum Ausdruck. Nach 48 Stunden war der Unterschied in der Wirkung der verschiedenen Verbindungen noch ausgeprägter. Bei den nur mit DNP behandelten Eiern war nach diesem Zeitpunkt Zytolyse eingetreten. In den übrigen Kolonnen ist der Zustand der zur weiteren Entwicklung gekommenen Eier in der % Anzahl der Blastulae angegeben. In den Lösungen mit Guanin, Hypoxanthin, Adenosin und Guanylsäure hatte sich nur eine geringe Anzahl Blastulae entwickelt. Dagegen zeigte sich in Hefeadenylsäure, Pyrophosphat und Muskeladenylsäure eine ansteigende Entwicklung der Blastulae, wobei auch wieder die Unterschiede in den einzelnen Konzentrationen hervortraten. Das beste Ergebnis war bei ATP zu verzeichnen.

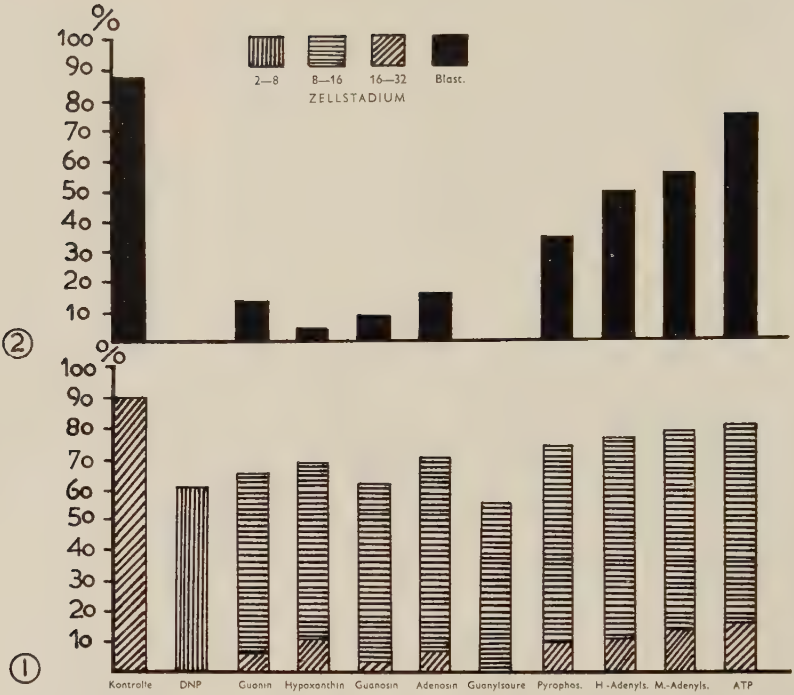


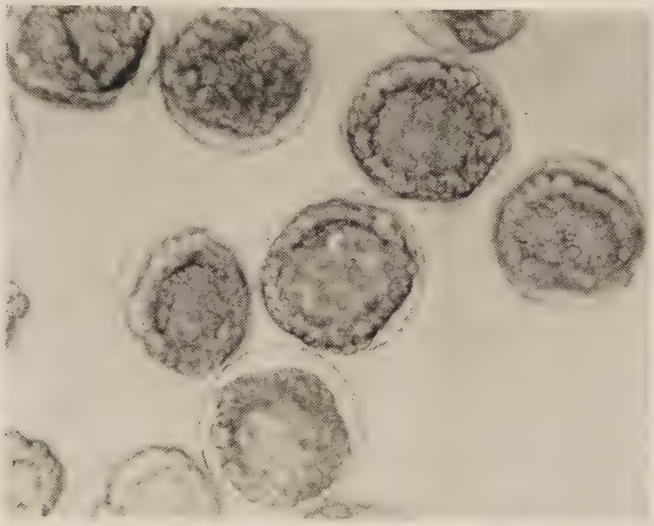
Diagramm 2. Entwicklungszustand der Eier von *Echinus*. 60 Min. nach der Befruchtung mit $5 \cdot 10^{-4}$ M DNP behandelt. 1. Nach 16 Stunden. 2. Nach 2 Tagen.

Hier war die Entwicklung sowohl in quantitativer als auch in qualitativer Hinsicht am weitesten fortgeschritten.

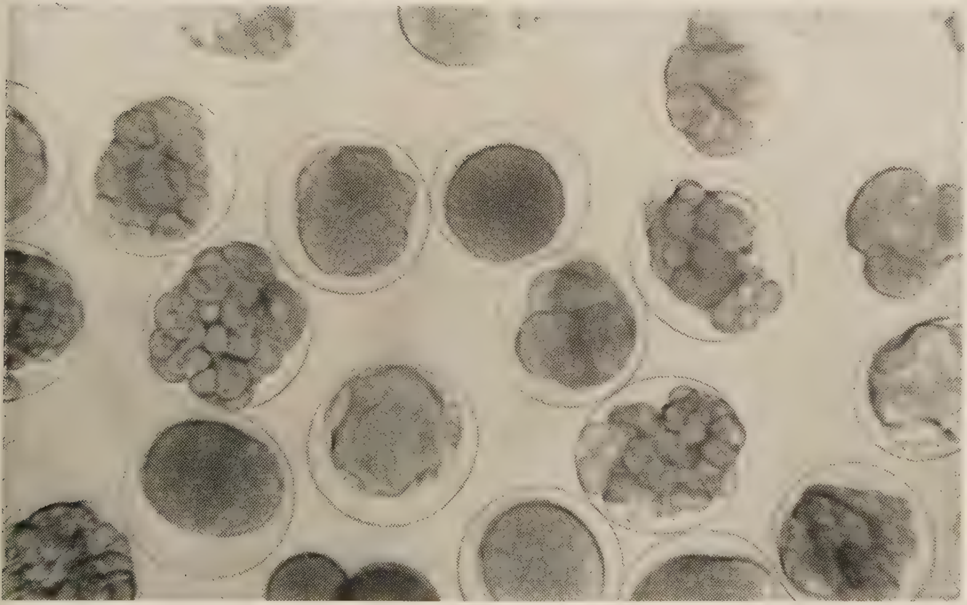
Die Abbildungen 1–6 verdeutlichen in einer Auswahl den in Diagr. 1, I zur Darstellung gebrachten Zustand der Eier nach 24 Stunden. Es ist erkennbar, dass die Eier in den einzelnen Verbindungen sowohl in bezug auf die Anzahl als auch auf den Zustand der Teilung wesentliche Unterschiede aufweisen. Die in ATP überführten Eier zeigen den besten Zustand auch in bezug auf die Regelmässigkeit der Teilungsstadien.

In Versuchen mit *Echinus* wurden die gleichen Verbindungen in einer Konz. von 0,004 M geprüft. In diesem Fall wurden die Eier 1 Stunde nach der Befruchtung mit einer Konz von $5 \cdot 10^{-4}$ M DNP behandelt und danach auf die verschiedenen Lösungen übertragen. Es ergaben sich hier ähnliche Unterschiede in bezug auf die Wirkung der einzelnen Substanzen wie bei *Strongylocentrotus*. Diagr. 2 zeigt den Zustand der Entwicklung in den verschiedenen Verbindungen nach 16 Stunden und nach 2 Tagen. Die Unter-

Entwicklungszustand der Eier von *Strongylocentrotus* nach Behandlung mit $2{,}5 \cdot 10^{-4}$ M DNP und Überführung in Adenosin, Hefeadenylsäure, Muskeladenylsäure und ATP.

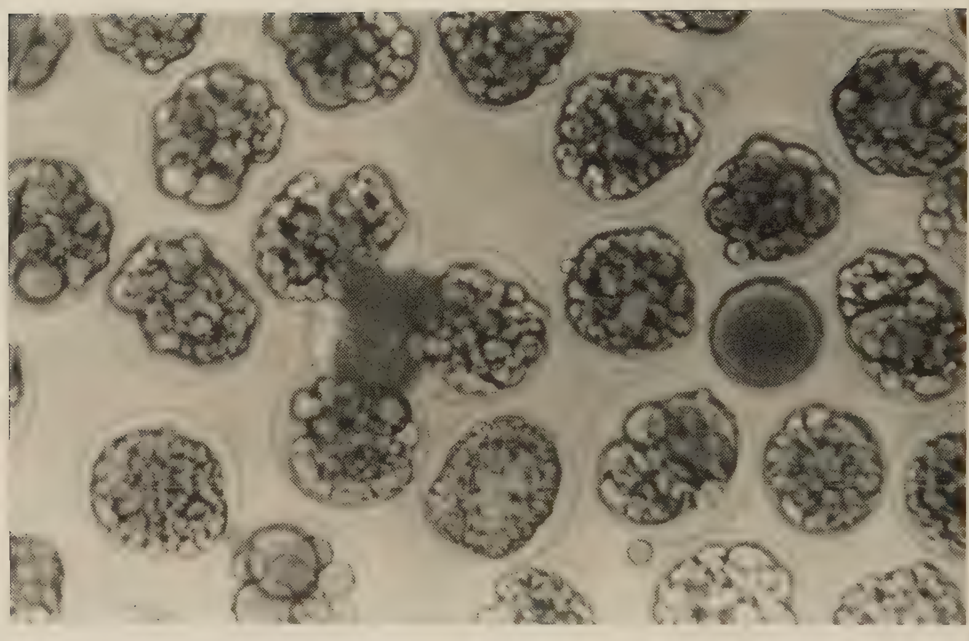


1. Kontrolle.

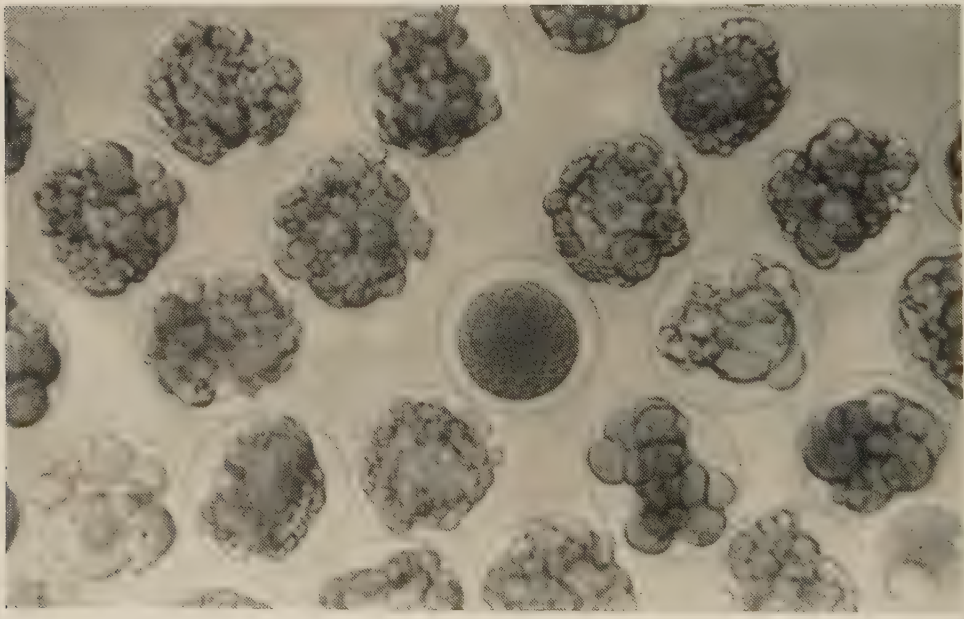


2. DNP.

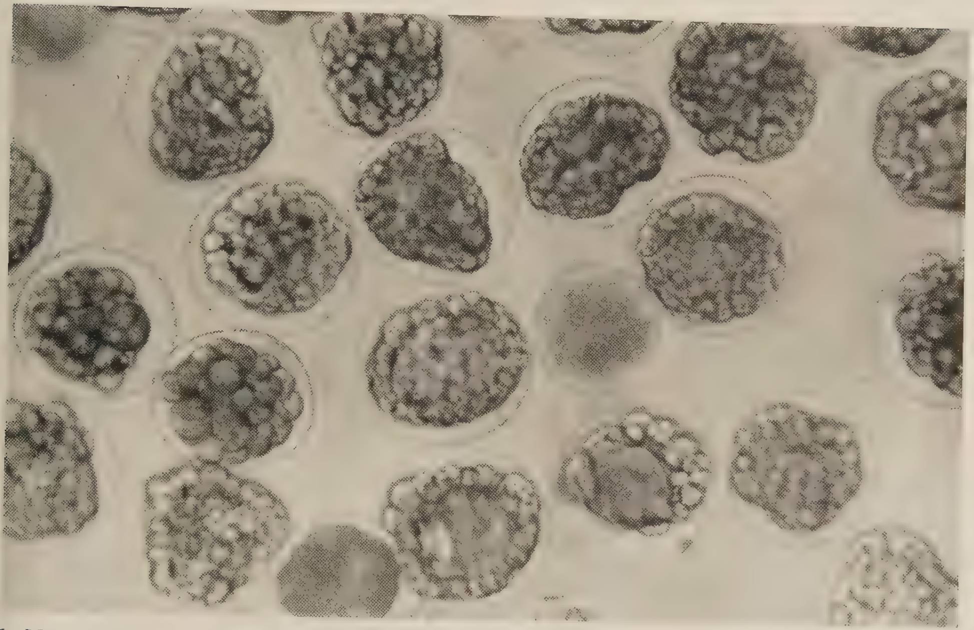
schiede im Zustand der Entwicklung sind hier bei den Adenylsäuren und Pyrophosphat nicht so stark abgestuft wie bei den Versuchen mit *Strongylocentrotus*. Auch in diesen Versuchen hatte ATP die beste Wirkung in Hinsicht auf die weitere Entwicklung der Eier.



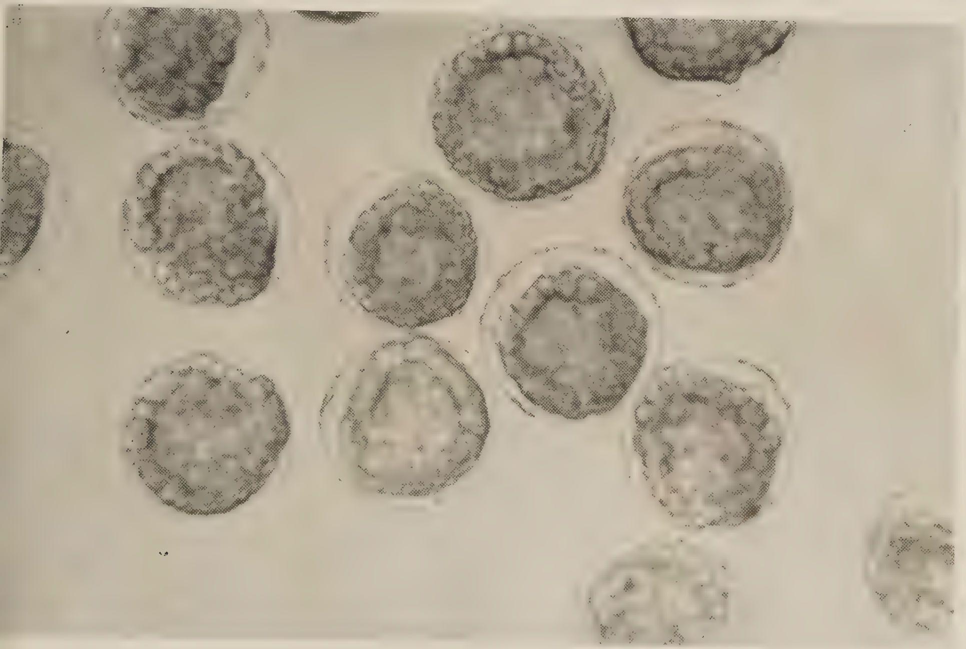
3. Adenosin 0,004 M.



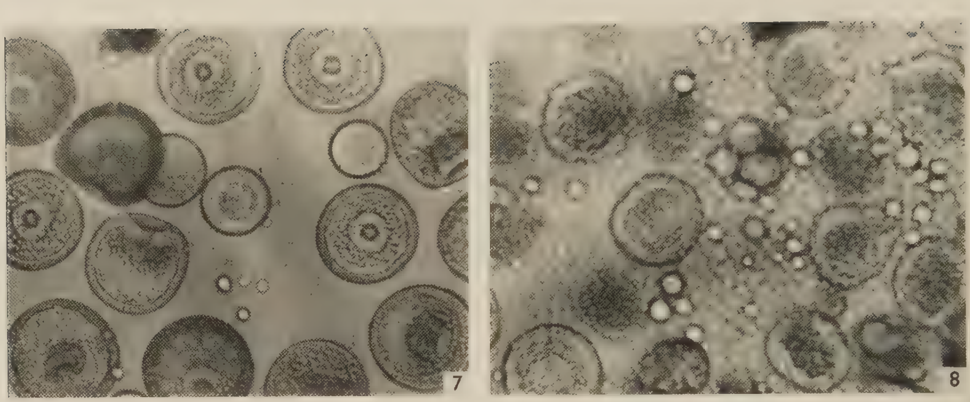
4. Hefeadenylsäure 0,004 M.



5. Muskeladenylsäure 0,004 M.

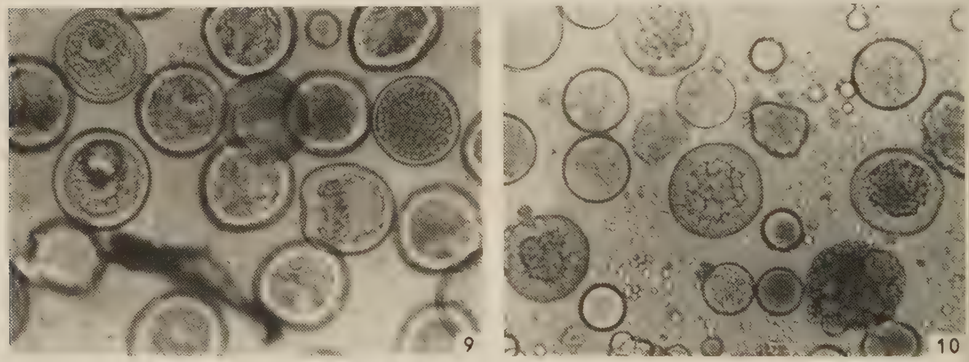


. ATP 0,004 M.



7. Kontrolle nach 4 Tagen.

8. Mit DNP behandelte Eier nach 4 Tagen.

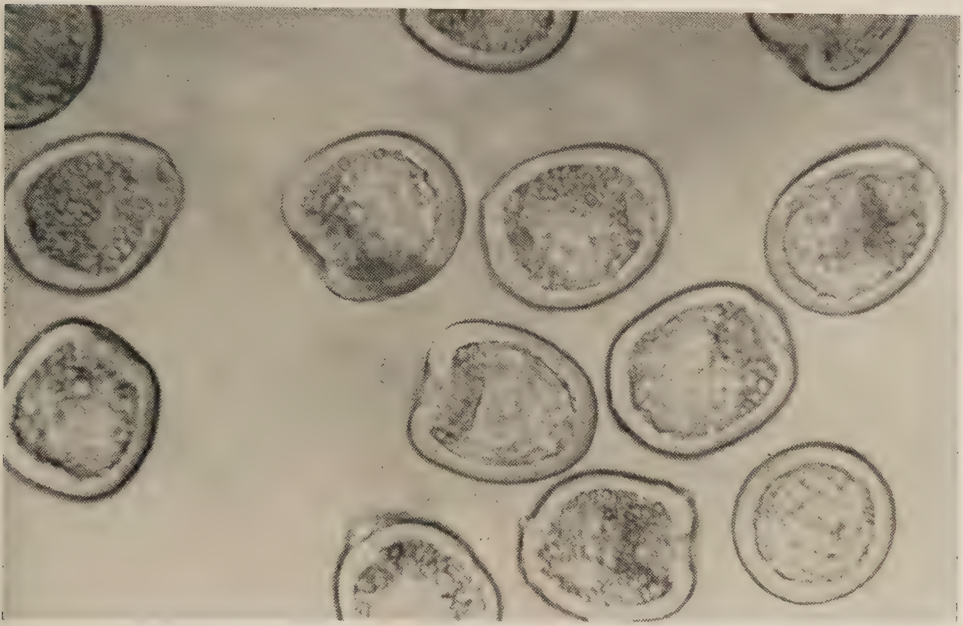


9. Mit DNP behandelt und in ATP überführt (nach 4 Tagen).

10. Mit DNP behandelt und in Hefeadenylsäure überführt (nach 4 Tagen).

Normale Blastulae von *Echinus* nach 2tägiger Entwicklung mit $5 \cdot 10^{-4}$ M DNP für 60 Min. behandelt und dann in 0,004 M ATP und in 0,004 M Hefeadenylsäure übertragen.

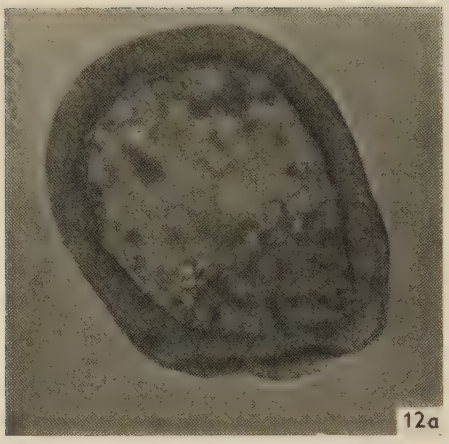
Wie aus weiteren Beobachtungen hervorging, entwickelten sich nur in den Lösungen mit Pyrophosphat sowie in den Adenylsäuren und ATP eine mehr oder weniger grössere Anzahl Blastulae, die im Verlaufe der weiteren Entwicklung auch zur Gastrulation kamen. Um zu erfahren, welchen Einfluss die Entfernung dieser Verbindungen auf die weitere Entwicklung hat, wurden die Eier nach einiger Zeit während der Entwicklung wieder in normales Seewasser überführt. In solchen Fällen zeigte sich nach der Übertragung in Seewasser ein deutlicher Entwicklungsstillstand, dem dann meist eine Zytolyse folgte. In Tab. I a und b sind Überführungsversuche dargestellt, aus denen hervorgeht, dass die Entfernung aus den betreffenden Verbindungen von wesentlichem Einfluss auf die Entwicklung ist.

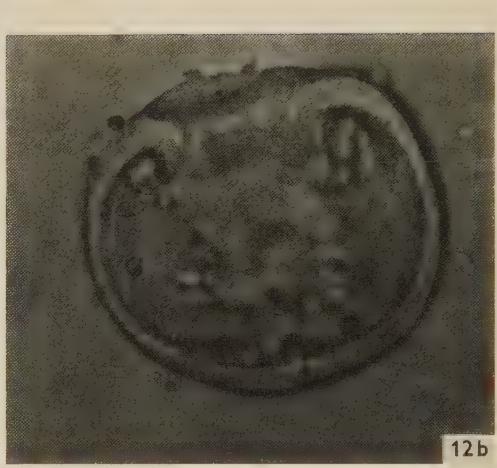


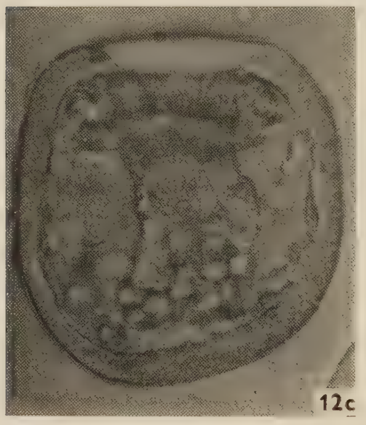
11. Zustand der Kontrolle vor der Behandlung mit DNP.

Blastulae von *Echinus* nach 4tägiger Entwicklung für 120 Min. mit $2{,}5 \cdot 10^{-4}$ M DNP behandelt und dann in ATP und in Seewasser überführt.

In weiteren Versuchen wurde geprüft, welchen Einfluss die Behandlung mit ATP oder mit Hefeadenylsäure hat, wenn die Entwicklung zu einem späteren Zeitpunkt der Entwicklung durch eine kurzfristige Behandlung mit DNP gehemmt wird. Zu diesem Zweck wurden die Eier von *Echinus* vor dem Beginn der Gastrulation 60 Min. mit $5 \cdot 10^{-4}$ M DNP behandelt. Ein Teil der Blastulae wurde dann in Seewasser, der andere in 0,004 M ATP und Hefeadenylsäure überführt. In diesem Fall ergab sich bei den in ATP übertragenen Blastulae ein wesentlich fortgeschrittener und besserer Entwicklungzustand im Vergleich mit den in Seewasser überführten mit DNP behandelten Blastulae. In Tab. II ist das Ergebnis dieser Versuche wiedergegeben. Die Abbildungen 11 und 12 a, b, c zeigen den Unterschied der Entwicklung von Blastulae, die mit DNP behandelt und danach gleichzeitig in ATP und in Seewasser überführt wurden. Die Abbildungen 7–10 zeigen den gleichen Versuch mit Hefeadenylsäure. In diesem Fall ist erkennbar, dass die in Hefeadenylsäure überführten Eier einen schlechteren Entwicklungszustand im Vergleich mit denen in ATP zeigen.







12. a. DNP behandelt und in Seewasser überführt.

12. b. DNP behandelt und in 0,004 M ATP überführt.

12. c. Unbehandelte Kontrolle.

Zustand der Blastulae nach der Behandlung.

BESPRECHUNG DER ERGEBNISSE

Die vorstehenden Versuche haben ergeben, dass die durch Behandlung mit DNP verursachten Hemmungen der Teilung und Entwicklung durch Überführung der Eier in Purine, Nucleoside oder Nucleotide in mehr oder weniger grossem Umfang aufgehoben werden können. Die Wirkung der Purine ist stark begrenzt. Es zeigte sich, dass hier nur am Anfang der Behandlung eine grössere Anzahl fortgeschrittener Teilungen auftrat im Vergleich mit den Eiern, die nur mit DNP behandelt worden waren. Dagegen war kein Einfluss der Purine auf die spätere Entwicklung sichtbar. Adenosin (bei *Strongylocentrotus* auch Guanylsäure) ergab im allgemeinen ein etwas besseres Resultat als die Purine. Aber auch hier war die Wirkung begrenzt, wie sich aus dem Vergleich der in Diagr. 1 und 2 dargestellten Befunde zeigt. Eine deutlich verbessernde Wirkung auf die Entwicklung über das Blastulastadium hinaus zeigten die Adenylsäuren, Pyrophosphat und ATP. Unter diesen nimmt ATP eine Sonderstellung ein, da es die Entwicklung sowohl in Hinsicht auf die Anzahl als auch auf die Regelmässigkeit wesentlich ausgeprägter fördert als die übrigen Verbindungen. Sowohl die Adenylsäuren als auch Pyrophosphat und ATP haben verschieden starke Wirkungen auf den Verlauf der späteren Entwicklung, wie aus den Versuchen hervorgeht. Diese Wirkung wird von der jeweiligen Konzentration bestimmt. Dabei zeigt sich sowohl eine obere Grenze, bei der die Verbindungen anfangen schädlich zu wirken, sowie ein Optimum und eine untere Grenze, bei der sie aufhört. In Versuchen von Barnett (1) bewirkte Adenylsäure oder unorganisches Phosphat keine Verbesserung der Teilungsquote bei *Arbacia*-Eiern, deren Teilung durch Sauerstoffmangel gehemmt war. Die dabei verwendeten Konzentrationen waren geringer als die in der vorliegenden Arbeit. Vermutlich konnte aus diesem Grund kein positives Resultat erzielt werden,

TABELLE I a

Eier von *Echinus esculentus* unmittelbar nach der Befruchtung für 60 Min. mit $5 \cdot 10^{-4}$ M DNP behandelt und danach in 0,004 M ATP und in 0,004 M Muskeladenylsäure übertragen. Die Hälfte der in ATP und in Muskeladenylsäure befindlichen Eier wurde nach 4 Stunden in Seewasser überführt.

		Kontrolle	DNP	Muskeladenyls.	ATP
I.	Zustand der nicht überführten Eier nach 20 Stunden.	95% frühe Blastulae mit gut entwickeltem Blastocoel.	30 % 8–16 und 20 % 16–32 Zellstadien.	60% frühe Blastulae mit unregelm. entwickeltem Blastocoel.	70 % meist gut entwickelte Blastulae.
II-	Zustand der nach 4 Stunden in Seewassser übertragenen Eier nach 20 Stunden.			50% fortgeschrittene Teilungsstadien ohne Blastocoel.	65 % Zellstadien über 32 Zellen mit schlecht entwickeltem Blastocoel.
I.	Zustand nach 2 Tagen.	90 % gut entwickelte Blastulae.	Zytolyse.	50 % Blastulae teilweise unregelmässig.	60 % meist gut entwickelte Blastulae.
II.	Zustand nach 2 Tagen.			Zytolyse.	Zytolyse.

TABELLE I b

Eier von *Echinus esculentus* wie oben mit DNP behandelt und danach in 0,004 M Hefeadenylsäure, Muskeladenylsäure, Pyrophosphat und ATP übertragen. Die Hälfte der in diesen Lösungen befindlichen Eier wurde nach 3 Tagen in Seewasser überführt.

		Kontrolle	DNP	Hefeadenyls.	Muskeladenylsäure	ATP	Pyrophosphat
I.	Zustand der nicht überführten Eier nach 3 Tagen.	90 % Blastulae.	Fortgeschrittene Teilungsstadien im Zustand der Zytolyse.	55 % Blastulae.	60 % Blastulae.	80 % Blastulae.	40 % Blastulae.
II.	Zustand der nach 3 Tagen in Seewasser überführten Eier nach 4 Tagen.		Zytolyse.	Zytolyse.	Meist Zytolyse.	Keine weitere Entwicklung. Meist abweichende Formen.	Meist Zytolyse.
I.	Nach 4 Tagen.	85 % Blastulae mit beginnender Gastrulation.		50 % Blastulae mit teilweise beginnender Gastrulation.	50 % Blastulae mit teilweise beginnender Gastrulation.	70 % Blastulae mit beginnender Gastrulation.	30 % Blastulae.

Bei I. nach 5 Tagen: fortschreitende Entwicklung. Bei II. nach 5 Tagen: auch in ATP Zytolyse.

da es sich in den vorliegenden Versuchen zeigte, dass die Wirkung von Adenylsäuren und Pyrophosphat bereits bei einer Konz. von 0,001 M wesentlich abnimmt.

In allen Versuchen zeigte sich, dass eine dauernde Einwirkung von ATP notwendig ist, wenn die durch DNP hervorgerufene Hemmung aufgehoben werden soll. Nach Überführung der in ATP befindlichen Eier in normales Seewasser trat eine erneute Entwicklungshemmung mit anschliessender Zytolyse auf. Das Gleiche konnte in Versuchen mit Pyrophosphat und Adenylsäuren beobachtet werden.

Wurde die Entwicklung zu einem späteren Zeitpunkt durch kurzfristige Behandlung mit DNP gehemmt, so liess sich ebenfalls durch Behandlung mit ATP eine weitgehende Verbesserung erzielen. Hefeadenylsäure zeigte in diesem Fall eine wesentlich schwächere Wirkung.

Die vorliegenden Ergebnisse haben gezeigt, dass die Zufuhr von ATP für die Wiederherstellung der durch DNP gehemmten Zellteilung und Ent-

TABELLE II

Normale Blastulae nach 2tägiger Entwicklung mit $5 \cdot 10^{-4}$ M DNP für 60 Min. behandelt und dann in 0,004 M Hefeadenylsäure und ATP übertragen.

	Kontrolle	DNP	Hefeadenylsäure	ATP
Zustand nach 4 Tagen.	90 % Blastulae mit beginnender Gastrulation. Abb. 7	Beginnende Zytolyse. Abb. 8	Meist abweichende nicht normale Formen und teilweise beginnende Zytolyse. Abb. 10	80 % Blastulae teilweise mit beginnender Gastrulation. Abb. 9

Normale Blastulae nach 4tägiger Entwicklung mit $2{,}5 \cdot 10^{-4}$ M DNP für 120 Min. behandelt und dann in 0,004 M ATP übertragen (Zustand der Blastulae Abb. 11).

	Kontrolle	DNP	ATP
Zustand nach 5 Tagen.	Ca 90 % Blastulae mit fortgeschrittner Gastrulation. Abb. 12 c	Durchgehend nicht gastrulierte Blastulae. Teilweise beginnende Zytolyse. Abb. 12 a	Meist Blastulae mit beginnender Gastrulation. Abb. 12 b

wicklung von wesentlicher Bedeutung ist. Es könnte angenommen werden, dass auch die Zufuhr von Pyrophosphat oder Adenylsäuren zum Aufbau von ATP in der Zelloberfläche oder im Zellinneren beitragen kann. Die wesentlich bessere Wirkung der Adenylsäuren und des Pyrophosphats auf die weitere Entwicklung im Vergleich mit den übrigen in diesen Versuchen verwendeten Verbindungen lässt eine solche Annahme möglich erscheinen. Dagegen kann Guanylsäure offenbar nicht in der gleichen Weise nutzbar gemacht werden, da es eine begrenzte Wirkung zeigte.

Die Empfindlichkeit der Eier der einzelnen Seeigelarten gegenüber DNP ist verschieden. Ausserdem ist die Konzentration der Lösung und die Dauer der Behandlung bestimmend. Bei *Paracentrotus lividus* erhielt Hörstadius (3) durch 2–4stündige Behandlung mit ca. 10^{-3} M DNP-Lösungen eine Vegetativisierung der Larven. Nach eigenen Versuchen bewirkt bei Eiern von *Psammechinus* 1–2stündige Behandlung mit 10^{-5} M DNP eine Hemmung der Teilung ohne eine wesentliche Schädigung der späteren Entwicklung. Wie aus den Ergebnissen von Clowes und Krahl (2) hervorgeht, verursacht 10^{-5} M 4,6-Dinitro-o-Kresol eine starke Zunahme des Sauerstoffverbrauchs der Eier von *Arbacia*, dagegen hemmen Konzentrationen von 10^{-4}–10^{-3} M die Atmung (siehe (2), Fig. 2, S. 153). Höhere Konzentrationen greifen demnach erheblich stärker in den Stoffwechsel ein und haben vermutlich auch eine mehr zerstörende Wirkung auf den Normalzustand der Eier. Wahrscheinlich beeinflusst die Behandlung mit 10^{-4} M DNP in den hier vorliegenden Versuchen nicht nur den Stoffwechsel der Eier, sondern es kommt darüber hinaus auch noch zu strukturellen Änderungen im Zustand des Zytoplasmas, besonders vielleicht der partikulären Komponenten desselben. Auf der anderen Seite kann ATP nicht nur in die durch DNP verursachten metabolischen Schäden regenerierend eingreifen, sondern es könnte auch in der Lage sein, wiederherstellend bei den oben erwähnten Veränderungen des Zytoplasmas mitzuwirken. Dass dem ATP im Hinblick auf das Zytoplasma solche Eigenschaften zukommen können und dass es einen Effekt auf den Zustand desselben hat, konnte in früheren Versuchen gezeigt werden (12). Runnström und Kriszat (11) konnten nachweisen, dass ATP die Teilungstendenz der Eier verbessert, so dass Eier, die vor der Befruchtung mit ATP behandelt wurden, sich besser teilten als solche in unbehandelten Kontrollen, bei denen der Zustand nicht vollständig normal war, so dass die Teilung unter der Normalquote lag. Bei Anwendung von Hemmungsfaktoren wie z. B. Behandlung der Eier mit hypotonischem oder hypertonischem Seewasser, das die Teilung ganz oder teilweise verhindert, trat diese Erscheinung noch deutlicher hervor (5).

Auch bei Versuchen, in denen Heparin (11) oder Colchicin (7) eine Hemmung der Teilung und Entwicklung der Eier bewirkte, verbesserte ATP nicht nur die Teilungsquote, sondern es verursachte auch eine regelmässigere Entwicklung der Eier. Diese Befunde können zur Erklärung der hier beobachteten Ergebnisse beitragen. Eine tiefere Analyse der Probleme des Stoffwechsels, die mit diesen Fragen in Zusammenhang stehen, liegt nicht im Rahmen dieser Arbeit. Ebenso bedarf die hier aufgezeigte Wirkung der Purine und der übrigen phosphatfreien Verbindungen noch der weiteren Aufklärung.

ZUSAMMENFASSUNG DER ERGEBNISSE

1. Bei Eiern von *Strongylocentrotus* und *Echinus*, die nach der Befruchtung kurzfristig mit $2{,}5$–$5 \cdot 10^{-4}$ M 2,4-Dinitrophenol behandelt wurden, konnte durch Überführung in verschiedene Purine sowie in Nucleoside und Nucleotide eine mehr oder minder ausgeprägte Aufhebung der durch DNP verursachten Hemmung der Teilung und Entwicklung bewirkt werden.

2. Die Behandlung mit Guanin oder Hypoxanthin sowie mit Adenosin, Guanosin oder Guanylsäure verursachte eine Verbesserung der ersten Teilungsstadien.

3. Pyrophosphat sowie Hefe- und Muskeladenylsäure und ATP verursachten eine weitergehende Aufhebung der durch DNP bewirkten Entwicklungshemmung.

4. Die Wirkung des Pyrophosphats und der Adenylsäuren war unterschiedlich und weniger ausgeprägt als die von ATP.

5. Bei Eiern, die im Zustand fortgeschrittener Teilungsstadien durch kurzfristige DNP-Behandlung geschädigt werden, bewirkt Überführung in ATP gleichfalls eine Verbesserung der späteren Entwicklung.

Am Ende dieser Arbeit ist es mir eine angenehme Pflicht, Herrn Prof. John Runnström für die Anregung zu den vorliegenden Untersuchungen und seine ständige Hilfe meinen allerherzlichsten Dank auszusprechen. Herrn Laborator O. Lindberg und Assistent L. Ernster bin ich für wertvolle Diskussionen zu Dank verpflichtet. Weiter danke ich Herrn Prof. Nils Holmgren und Herrn Dr. Gunnar Gustafson von der Zoologischen Station Kristineberg für Ihre freundliche Hilfe und die ständige Beschaffung des für die Untersuchungen benötigten Materials.

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THE EFFECT OF DEOXYRIBONUCLEIC ACIDS EXTRACTED FROM SEA URCHIN SPERM ON THE DEVELOPMENT OF SEA URCHIN EGGS

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In 1949, two of the authors carried out some preliminary experiments at the Marine Biological Laboratory, Plymouth, in which nucleic acids were injected into the eggs of the sea urchins *Echinus esculentus* and *Psammechinus miliaris.* Deoxyribonucleic acid (DNA) extracted from calf thymus and ribonucleic acid (RNA) extracted from yeast were injected into unfertilized and fertilized eggs. The effect of these substances was, on the whole, similar. The injected fluid either remained as a distinct droplet (Harvey (5) referred to this droplet as an artificial nucleus) or mixed with the cytoplasm. In the former case, unfertilized eggs could be fertilized, whereupon they raised membranes and developed into plutei; injected fertilized eggs also developed normally. The droplet of injected fluid as a rule moved slowly toward the surface of the egg and was pushed out, either before segmentation, or from a blastomere at an early or late cleavage stage. In one case, such a droplet remained in a large cell which projected into the blastocoele and was later found lying free in the blastocoele. In many of the eggs, however, the injected fluid had an injurious effect even though the droplet remained discrete. In such cases several asters formed but no cell division took place before the eggs cytolysed. Sometimes only a part of an egg showed such injury effects, whereas the greater part passed through segmentation and developed into gastrulae or plutei. When, on the other hand, the injected fluid mixed with the cytoplasm of an unfertilized egg, this egg became activated and immediately raised a high membrane; cytolysis soon followed, however.

Largely because of the importance of DNA as a constituent of chromatin, Mazia (18) has sought for a method to detect species differences between preparations of DNA. According to Mazia, eggs of *Rana pipiens* were not affected by DNA from ox, but were arrested at the yolk plug stage by DNA from their own species. Similarly, eggs of the starfish *Asterias forbesii* developed normally in DNA extracted from *Arbacia punctulata* and *Echinarachnius parma* but were prevented from gastrulating by *Asterias* DNA.

The aim of the present investigation was to test whether DNA from the sperm of different species of sea urchins (preparations of Chargaff (2)) would show any species specific differences as to the time from injection-activation to the onset of cytolysis, and also whether eggs might possibly be affected in any special way when developing in media containing different kinds of DNA.

Since DNA is believed to occur only in the nucleus, it might seem strange to suppose that treatment with DNA should have any effect. Mazia, however, (18) has supplied evidence that in the early stages of development the enzyme DNAase is located also in the cytoplasm of the sea urchin egg. He mentioned two alternatives: 1) the enzyme may be spatially separated from its substrate DNA, which is believed to be exclusively nuclear, or 2) the localization of the enzyme may indicate that its substrate DNA is also largely cytoplasmic, at least in the early stages of development. Zeuthen (20) compared analytical data for total and nuclear DNA in sea urchins and emphasized that the latter possibility is more likely. Later, Hoff-Jørgensen and Zeuthen (7) separated the germinal vesicle from the cytoplasm of frog oocytes and demonstrated that the latter contained considerable quantities of DNA, greatly in excess of that present in the nucleus. Most of the cytoplasmic DNA appeared to be a relatively low polymer. The view that DNA is stored in the cytoplasm during early developmental stages is supported by: 1) Agrell's (1) observation that sea urchin eggs show a clear coloration of the cytoplasm with Gomori's haematoxylin during the first 4 hours of segmentation (this stain is more intensive than the Feulgen reaction), and 2) Elson and Chargaff (4) found about 25 times as much DNA per unfertilized egg of *Paracentrotus lividus* as per sperm cell of the same species.

MATERIAL

The experiments were performed at the Station Biologique, Roscoff, in the summers of 1950 and 1951 on freshly shed eggs of *Paracentrotus lividus* and *Psammechinus miliaris.*

METHODS

Preparation of DNA. The DNA preparations were extracted from the sperm of *Paracentrotus lividus, Psammechinus miliaris, Arbacia lixula,* and *Echinocardium cordatum* as described by Chargaff *et al.* (2), except that the final extracts were not subjected to lyophilization, but were stored in fibrous form in aqueous alcohol.

Analytical data for all of the extracts used have been presented in the above publication.

The DNA preparations were kept in 66 per cent alcohol. After drying in a dessicator over calcium chloride, a certain amount was weighed and dissolved in 9 ml 0.2 M KCl ·1 ml 0.2 M $MgCl_2 \cdot 6\ H_2O$ (Callan, personal communication). When all the DNA was dissolved (stirring and slight warming was necessary), the pH was adjusted with KOH to about 7. A 1 per cent solution gave a rather rigid jelly, and a 0.5 per cent solution was more or less viscous. The DNA from different species showed differences in this respect. The solutions were used immediately, dilutions being made with the salt solution for injections or with sea water for rearing experiments.

For injections eggs were placed in hanging drops of filtered sea water in a paraffin oil chamber. The de Fonbrune Micromanipulator was used. In order to ensure mixing of the DNA with the cytoplasm of the egg, it was found useful to withdraw a little cytoplasm into the micropipette and then immediately reinject the cytoplasm with the DNA solution. The latter is then seen to diffuse into the cytoplasm. In cases where the DNA formed a distinct droplet and did not mix with the cytoplasm, no activation was obtained in unfertilized eggs, and the DNA as a rule was sooner or later extruded from the egg, as mentioned in the introduction. Such cases are not included in the results. The volume injected was estimated as being from 1 to 6 times that of the nucleus.

RESULTS

Development of whole eggs in DNA solutions

Fertilized eggs were placed in solution of DNA diluted with sea water; pH adjusted to about 8.2.

DNA of Paracentrotus. Eggs of *Paracentrotus* were able to develop both in 0.5 per cent and 0.25 per cent solutions, in spite of the high viscosity of the 0.5 per cent solution, which almost prevented movement. But the larvae started to swim normally when transferred to sea water. Many larvae died, but in 0.5 per cent some reached the gastrula-prism stage. Those which were transferred to sea water at this stage developed into normal plutei. In the 0.25 per cent solution some larvae reached the pluteus stage.

Eggs of *Psammechinus* gave somewhat similar results. In 0.5 per cent the eggs did not survive the gastrula stage, but those transferred to sea water at the blastula stage developed into normal plutei. In 0.25 per cent also, some of those eggs which had been left in the solution all the time reached the pluteus stage although their arms remained short.

DNA of Psammechinus. Eggs of *Paracentrotus* in 0.5 per cent solution showed some abnormalities during segmentation and were more or less filled with dark cells at the blastula stage. They did not survive longer, whereas those which were transferred to sea water at the early blastula stage developed into plutei. In the 0.25 per cent solution cleavage was normal, but development was arrested at early gastrula stages. Those introduced into sea water as blastulae differentiated into normal plutei. In 0.125 per cent and 0.025 per cent the development went further, to a stage corresponding to the prism stage, but with skeletal development poor or lacking.

Eggs of *Psammechinus* in 0.5 per cent solutions cleaved in a more normal manner than the *Paracentrotus* eggs but died earlier. In 0.25 per cent cleavage was normal, and the larvae reached the mesenchyme blastula stage, but did not gastrulate. Those transferred into sea water at the blastula stage advanced, however, to normal plutei. In 0.125 per cent gastrulation began, and in 0.025 per cent some abnormal prism stages were obtained.

DNA of Arbacia lixula. Eggs of *Paracentrotus* were able to cleave normally in the viscous 1 per cent solution and even reach the stage of beginning gastrulation. Blastulae transferred to sea water developed into normal plutei. Some larvae in 0.5 per cent attained a differentiation corresponding to the pluteus stage but showed some indications of radial symmetry regarding ciliated band and skeleton, although the digestive tract was bilateral as far as the stomodaeum and oesophagus were concerned. Some larvae which had been transferred to sea water as mesenchyme blastulae showed the same type of abnormality whereas others differentiated into normal plutei. Some of these radial types occurred also in 0.25 per cent solutions.

In 1 per cent solution, eggs of *Psammechinus* also showed a normal early cleavage, but the blastomeres cytolysed in later cleavage stages. In 0.5 per cent a few larvae developed into prisms or young plutei, without transfer, and in 0.25 per cent many plutei appeared.

DNA of Echinocardium cordatum. 0.5 per cent solutions of *Echinocardium* DNA were too injurious to *Paracentrotus* eggs. Only one or two cleavages took place. In 0.25 per cent solution the blastulae in one series looked normal, but the following day all were dead; in another series some reached the pluteus stage. With eggs from the same female as the first mentioned series, 0.125 per cent allowed development to the prism stage, and in 0.025 per cent plutei were obtained.

The eggs of *Psammechinus* also stopped developing after one or two cleavages in 0.5 per cent. In 0.25 per cent they died as early blastulae in one series; in another series the eggs (from a second female) died as late blastulae, and, in a third series, some began to gastrulate. The degree of inhibition was about the same in 0.125 per cent, but in 0.025 per cent some plutei were observed.

Conclusions regarding the development of eggs in DNA solutions. Fertilized eggs may develop even in strong concentration of DNA prepared from sea urchin sperm. The findings of Mazia (18) that the development of *Rana pipiens* and *Asterias forbesii* is arrested by DNA from the same species but not from others could not be confirmed with our material. Eggs of *Paracentrotus* developed into larvae in strong solutions of *Parac.* DNA: in fact, their development was even better under these conditions than that showed by the eggs of *Psammechinus miliaris.* In *Psamm.* DNA the development of eggs of both these species was about the same (*Psamm.* larvae possibly slightly more inhibited).

On the other hand, the DNA of the less related irregular sea urchin *Echinocardium* was more injurious than those of the regular sea urchins *Paracentrotus, Psammechinus,* and *Arbacia.*

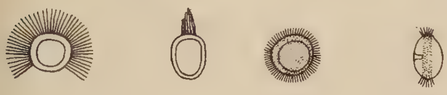
The conclusion that the DNA of sperm from the same species is not more injurious than DNA from other sources is further strengthened by the results on the rearing of animal halves (see next section).

The presence of DNA of another species had no effect on the species character in that the skeletal rods in neither *Parac.* nor *Psamm.* larvae showed any sign of forming fenestrated rods in DNA of *Echinocardium,* in whose ontogeny this trait is observed.

Animal halves treated with DNA

The majority of isolated animal halves in pure sea water show a more or less enlarged apical tuft, covering up to about three-fourths of the surface of the blastula (type $\geqq \frac{1}{2}$, Fig. 1). (Isolated animal halves do not gastrulate.) Other have a smaller apical tuft, and there are all kinds of intermediate stages down to some animal halves with normal apical tufts (type $\frac{1}{8}$). A couple of days later those with a large apical tuft develop into uniformly ciliated blastulae (type A) or into blastulae with only a small area of thin pavement epithelium (type Ba). Blastulae with intermediate apical tufts may give also type Ba, but often also, either a blastula with a ciliated field covering less than the half of the surface (type Bc) or even a blastula with a ciliated band (type C). Those with a small apical tuft ($\frac{1}{6}$ or $\frac{1}{8}$) as a rule differentiate into type C or into a blastula with both ciliated band and stomodaeum (type D). Elaborate series of experiments with gradual diminishing of vegetal material in eggs have given the explanation of the different behavior of animal halves. The enlargement of the apical tuft and the less marked differentiation of the animal half is due to lack of vegetal influences which are necessary in order to check the expansion of the apical tuft and to call forth the formation of ciliated band and stomodaeum (8, 9, 10, 11).

It seems probable that the animal and vegetal properties or principles, which have to interact in order to bring about a typical differentiation, are based on differences in metabolism. Eggs can be vegetalized or animalized by chemical means, as by lithium ions (6) or by thiocyanate ions (16). It is impossible to detect with certainty small changes in an animal or vegetal direction on whole eggs. Animal halves are more sensitive in this respect, and the size of the apical tuft and the degree of later differentiation are indicators of the strength of the vegetal principle in relation to the animal. Using isolated animal halves it has been possible to test the effect of many substances of more or less well-known physiological action (12–15). It is in a similar way that the influence of DNA has been tested on isolated animal halves.



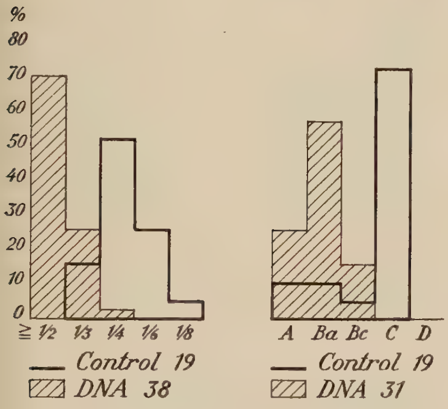


Fig. 1.

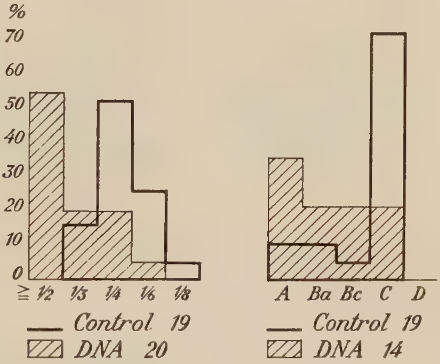


Fig. 2.

Fig. 1. Animalizing effect of DNA of *Paracentrotus lividus* on animal halves of the same species. The columns indicate the percentage of the different types. Column with heavy lines: control halves. Hatched columns: treated halves. Left row: frequency of types ≧ ½–1/8 on the first day after operation. Right row: frequency of types A–D the following day. Cf. text. The numerals indicate the number of control halves and treated halves.

Fig. 2. Animalizing effect of DNA of *Arbacia lixula* on animal halves of *Paracentrotus lividus*.

The diagrams (Figs. 1–3) show that the DNA from *Paracentrotus, Arbacia* and *Echinocardium* all had a marked animalizing effect on animal halves of *Paracentrotus*. The material is not large, but it seems clear that the treated halves as a rule show larger apical tuft on the first day and later a more uniform animal differentiation than observed in the controls. These experiments further emphasize that the DNA of the same species is not harmful.

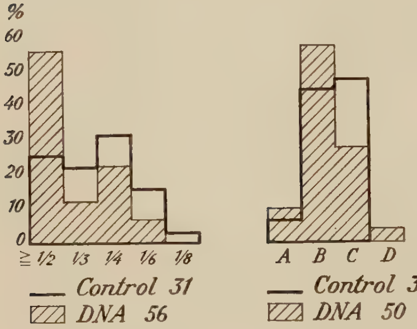


Fig. 3.

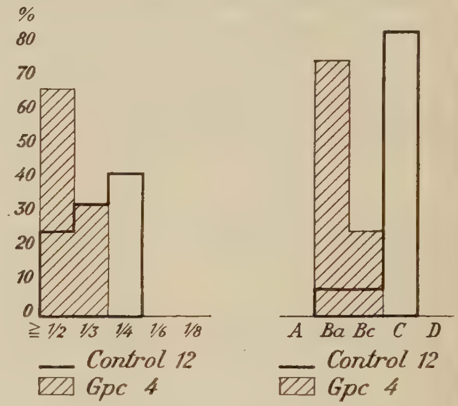


Fig. 4.

Fig. 3. Animalizing effect of DNA of *Echinocardium cordatum* on animal halves of *Paracentrotus lividus*.

Fig. 4. Animalizing effect of glucopyranosylcytosine HCl on animal halves of *Paracentrotus lividus*.

Animal halves treated with synthetic nucleosides

It may be of interest to mention in this connection that experiments with synthetic nucleoside solutions yielded similar results. The substances were kindly put at our disposal by Dr. Jack J. Fox and brought from the Laboratoire de Morphologie Animale, Brussels (courtesy of the Director, Professor Jean Brachet). The substances used were: 1. D-β-glucopyranosylcytosine hydrochloride, 2. D-β-xylopyranosylcytosine hydrochloride, 3. α-arabinopyranosylcytosine, 4. D-β-galactopyranosyluracil, and 5. D-β-galactopyranosylcytosine hydrochloride. The synthetic nucleosides 1, 2, 3, and 5 are structural analogues of cytidine; 4 is an analogue of uridine. These compounds differ from their natural analogues only with respect to the sugar moiety. Both cytidine and uridine are found in hydrolyzates of ribonucleic acid.

The solutions (in sea water) were 0.01 M and 0.005 M in four of the cases; number 3 was used at 0.002 M. *Paracentrotus* eggs were used. With 4 and 5 no change of differentiation was observed, but with the other substances there was a clear animalizing influence (Figs. 4–6), just as there was with

DNA. This effect is particularly well shown in Fig. 6, where the halves treated with 0.01 M are more animal than those from 0.005–0.002 M solutions, and these in turn more animal than the controls. The data are not extensive as the substances were available only in limited amounts which did not permit repitition of the experiments. The results seem worth mentioning, however, without too much emphasis on their interpretation.

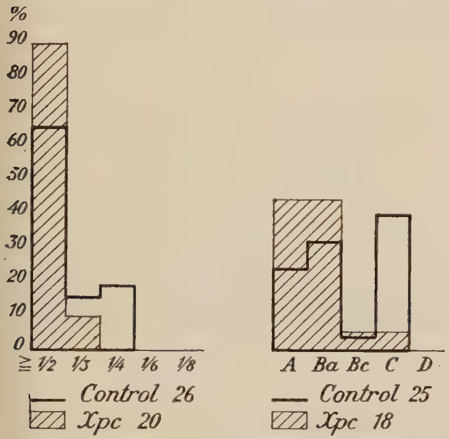


Fig. 5.

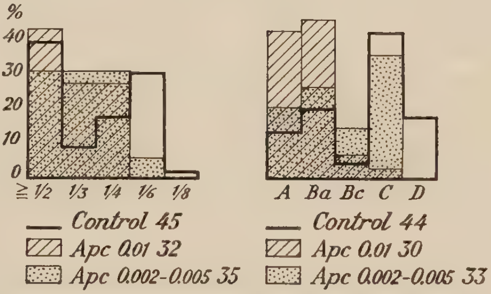


Fig. 6.

Fig. 5. Animalizing effect of xylopyranosylcytosine HCl on animal halves of *Paracentrotus lividus*.
Fig. 6. Animalizing effect of arabinopyranosylcytosine on animal halves of *Paracentrotus lividus*. Hatched columns: halves treated with 0.01 M, stippled columns with 0.002–0.005 M.

Results of micro-injection experiments

Since it proved impossible with our equipment to inject into the cytoplasm an accurately measured amount of the DNA solutions, the primary object of the investigation, a test for species differences was not attained. The records are therefore not presented in full. As controls, many unfertilized eggs were injected with the KCl-$MgCl_2$ solution. Upon subsequent fertilization, these control eggs raised normal membranes and normal plutei developed.

DNA of Paracentrotus. 0.5 per cent DNA. Seven unfertilized *Parac.* eggs were injected; three raised fertilization membranes, two partial membranes, and two did not respond. Cytolysis occurred in all cases, the time between injection and cytolysis varying between 15 minutes and 5 hours. Of nine injected *Psamm.* eggs, eight raised membranes; the one without a membrane received only a small volume of DNA solution. All had cytolysed in less than 45 minutes. The period between injection and membrane elevation was 30 seconds to 2 minutes in both species.

0.25 per cent DNA. Five out of nine *Parac.* eggs injected showed membrane elevation. All cytolysed in 20 minutes to 3 hours. Five of six *Psamm.* eggs raised membranes; the one that failed to do so received a very small volume of solution (approximately equal to the nuclear volume). All cytolyzed in less than 1 hour. The period between injection and membrane elevation was between 1 and 7 minutes in both species.

0.05 per cent DNA. Four *Parac.* and 3 *Psamm.* eggs were injected. None raised a membrane. Fertilization was attempted without success. All cytolysed in 2–6 hours.

Conclusions concerning the DNA of Paracentrotus. The lowest concentration used did not cause membrane elevation but prevented subsequent fertilization and caused cytolysis in both species. At higher concentrations (0.25 per cent and 0.5 per cent) nearly all of the *Psamm.* eggs injected raised membranes; only half of the *Parac.* eggs showed membrane elevation, however. In *Parac.* the time between injection and membrane elevation was very variable. The same applies to the time before signs of serious injury occurred (after a time, injected eggs showed visible signs of coagulation, such as a change in light-scattering properties; this will be referred to as the "coagulation time"). In *Psamm.* eggs the "membrane time" was definitely less when higher concentrations were injected (0.5 per cent), and the "coagulation time" varied inversely with the concentration of DNA. The *Psamm.* eggs coagulated more quickly than the *Parac.* eggs.

DNA of Psammechinus. A solution of 0.075 per cent was injected into four *Psamm.* eggs; these eggs elevated membranes and "coagulated" after about 90 minutes. With 0.0075 per cent, seventeen eggs showed no membrane elevation; six of these could be fertilized, and two developed: one into a dark colored blastula, and the other into a prism larva with small spicules. Four others were also injected, but the drop of solution ran out. After fertilization all developed into normal plutei.

All of the four *Paracentrotus* eggs injected with 0.25 per cent DNA elevated membranes. Three coagulated within 20 minutes, but one survived and showed aster-like radiations in the cytoplasm three hours after injection. It then divided into irregular lobes and lasted over 5 hours. The nucleus did not divide. Of five eggs injected with 0.75 per cent DNA, three raised membranes. All coagulated within an hour of injection. Three eggs injected with 0.075 per cent raised no membranes and died after more than 2 hours. With 0.0075 per cent only three out of fourteen injected eggs formed membranes. Two coagulated after 3 hours, but one egg divided into irregular lobes 4 hours after injection. The other eggs responded to fertilization by forming membranes; one blastula, two dark gastrulae and one pluteus were obtained. Six eggs were injected with 0.0015 per cent; these were subsequently fertilized and developed into plutei.

DNA of Arbacia. Two *Parac.* eggs injected with 1.0 per cent, five with 0.2 per cent and four with 0.25 per cent, all formed membranes, but survived only 30–60 minutes. With 0.075 per cent only two out of five and with 0.0075 none of five raised membranes. All of the latter group were subsequently fertilized and yielded two blastulae and three plutei. *Psamm.* eggs gave similar results. Two eggs with injections of 1.0 per cent formed membranes and then coagulated after 10 minutes. With 0.75, 0.1, and 0.075 per cent, membranes usually formed and the average survival time was 45 minutes. Injections of 0.0075 per cent did not cause membrane formation and the eggs did not coagulate until after 3 hours.

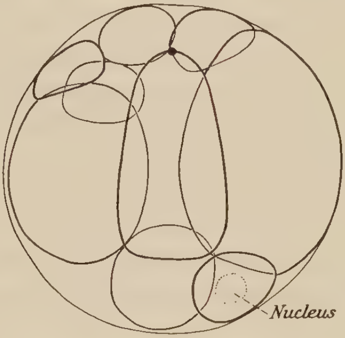


Fig. 7. Irregular cleavage stage of *Paracentrotus lividus* after cytoplasmic activation of unfertilized, mature egg by injection of 0.075 per cent of DNA of *Echinocardium cordatum.* $5^1/_2$ hrs after injection. Camera lucida drawing.

DNA of Echinocardium. Only *Paracentrotus* eggs were used; after injection of 75 and 0.075 per cent, all six eggs raised membranes and cytolysed after 1–8 hours. Twenty-three eggs were injected with 0.0075 per cent and only six raised membranes; these coagulated after about 4 hours. Most of the others were successfully fertilized and several developed into plutei.

No less than five of the twelve eggs which raised membranes after injection of 0.75 to 0.0075 per cent DNA showed signs of further development. In some cases the cytoplasm divided in a way resembling a cleavage although with cells of irregular size (Fig. 7). But sooner or later this cytoplasmic division resulted in increasingly irregular lobes, a process similar to the pathological cleavage observed in enucleated blastomeres (17). Neither aster formation nor nuclear division occurred. The nucleus could usually be seen lying in one of the cleavage products (Fig. 7). As mentioned above, the eggs coagulated after about 4 hours. However, the cytoplasmic activation leading to a stage similar to Fig. 7 is noteworthy. Of thirteen eggs injected with 0.0015 per cent only two formed membranes. The others were fertilized and the majority developed into plutei, and the remainder into dark blastulae or gastrulae.

DNA from calf thymus. Droplets of 0.75 per cent injected into seven eggs did not mix with the cytoplasm. Consequently the eggs could be fertilized and the drop eventually came out. Three plutei developed whereas the others only reached the gastrula or prism stages. One out of four eggs injected with 0.075 per cent elevated a membrane; all of these cytolysed after about 2 hours. Three out of seven eggs injected with 0.0075 per cent raised membranes, but survived only about 1 hour.

Conclusions concerning micro-injection experiments. Under dark-field illumination the surface layer of the sea urchin eggs changes from yellow to white during activation. The change is more or less visible depending upon the species used (19). This change of color was observed in unfertilized *Paracentrotus* eggs after injection with *Echinocardium* DNA and calf DNA (no other observations were made). In the latter case there were four injected and three control eggs in the same drop. The controls remained yellow whereas the injected ones turned clearly white; the other injected eggs showed an intermediate color. The activation therefore leads not only to membrane formation, but also to the typical change in the cortex which manifests itself in a color change under dark-field illumination.

A real cleavage never followed upon activation with DNA, but in six cases there were more or less abnormal attempts at cytoplasmic division (Fig. 7), finally resulting in a clump of pathological cells of irregular shapes and sizes. The nucleus always remained intact.

Activation by DNA was achieved only after injection, and never when unfertilized eggs were merely placed into strong solutions.

The volume of DNA injected varied between 1–6, as a rule 2–4 times the volume of the nucleus. The time between injection and membrane elevation was roughly inversely proportional to the volume injected, for a given concentration. The survival time, on the other hand, was not strictly correlated with the volume injected, but rather roughly correlated with the concentration injected. Injection of low concentrations often did not cause membrane elevation or cytolysis, and did not prevent subsequent fertilization and development.

Because the amount of fluid injected was not accurately measured, the question of species specificity could not be clearly answered. It might, however, be mentioned that according to our experience at Plymouth, and according to the experiments quoted above, calf thymus DNA seems to be more injurious than DNA extracted from sea urchin sperm. No such marked difference in toxicity was found between different sperm preparations.

The species specific skeletal development of *Paracentrotus* was not changed by injection of *Echinocardium* DNA.

SUMMARY

Eggs of *Paracentrotus lividus* and *Psammechinus miliaris* were reared in sea water with different concentrations of DNA from sperm of *Paracentrotus lividus, Psammechinus miliaris, Arbacia lixula* and *Echinocardium cordatum.* DNA from the same species did not arrest development. DNA from the irregular sea urchin *Echinocardium* seemed to be more injurious than the others.

Animal halves became more or less animalized when reared in solutions of DNA (Figs. 1–3) or in three of the five tested synthetic nucleosides (Figs. 4–6).

When the injected DNA did not mix with the cytoplasm the egg could be fertilized and develop normally; in these cases the drop was slowly ejected from the cell.

If DNA injected into unfertilized eggs mixed with the cytoplasm the stronger solutions caused activation. A fertilization membrane was elevated, a color change of the surface layer sometimes occurred, and in a few cases an abortive cleavage occurred (Fig. 7), but eventually the egg cytolysed. Eggs which did not become activated after injection of low concentrations of DNA could be fertilized, and developed normally. As the volume injected was not accurately measured, no positive conclusions can be drawn concerning species specificity of the DNA preparations used.

Neither treatment with, nor injection of *Echinocardium* DNA changed the species character of the larvae of *Paracentrotus.*

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Experimental Cell Research, **6**, *453–458* (*1954*)

THE EFFECT OF LECITHINASE A ON THE MORPHOLOGY OF ISOLATED MITOCHONDRIA

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THE purpose of this work was to investigate the morphological changes resulting from hydrolysis of mitochondria with lecithinase A. This problem was of interest for the following reason: Lecithinase A is known to inactivate some factors in the succinoxidase and in the DPNH-oxidase system (6, 7). These factors are believed to function as structural components, to keep the individual catalysts (cytochromes *b* and *c*, DPNH-cytochrome *c* reductase and cytochrome *c*) in the right spatial relationship. Consequently, inactivation of these factors may be expected to be associated with structural alterations of the mitochondria.

For comparison, the study with lecithinase A was extended to include the morphological effect of lysophosphatides (the products of the action of lecithinase A), and of lecithinase C and trypsin, which are known to inactivate the succinoxidase system (1, 4, 8).

MATERIALS AND METHODS

We used inbred albino rats. The homogenization of the liver was carried out as described in a previous paper (2). A 0.25 M sucrose solution was used throughout. Differential centrifugation of the homogenate was made in a Spinco preparatory centrifuge at 0°C. The sediment obtained after 15 minutes at 1500 × was discarded. The unwashed precipitate at 6600 × g was used for our experiments.

The mitochondrial suspensions were incubated with the reagents at 38° in a water bath. 1 ml of suspension contained the particles from roughly 0.5 g of liver tissue. After incubation, samples were taken for morphological investigations and for determinations of respiratory activity. Fixation of the particles was made by the addition of 0.1 ml of 1 per cent OsO_4 solution to 1 ml of the mitochondrial suspension.

The preparation of the specimens for the electron microscope was made according to the procedure outlined in a previous paper (2). The electron microscope was R.C.A. type EMU, provided with a 100 μ condensor aperture.

The succinoxidase activity was determined according to the method of Schneider and Potter (9).

Lecithinase A. The same crystalline preparation was used as employed in previous studies (6, 7). The purity of this preparation has been examined thoroughly (7).

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[1] Fellow Norwegian Research Council.

Lysophosphatides. A mixture of lysolecithin and lysocephalin was prepared from egg yolk by the method of Levene *et al.* (3).

Lecithinase C. This preparation was obtained through the courtesy of Dr. M. MacFarlane. It had been isolated from the toxin of *Clostridium welchii* and was reported to be contaminated with small amounts of Θ-toxin and hyaluronidase. The enzymatic activity of lecithinase C was 25 units per mg.

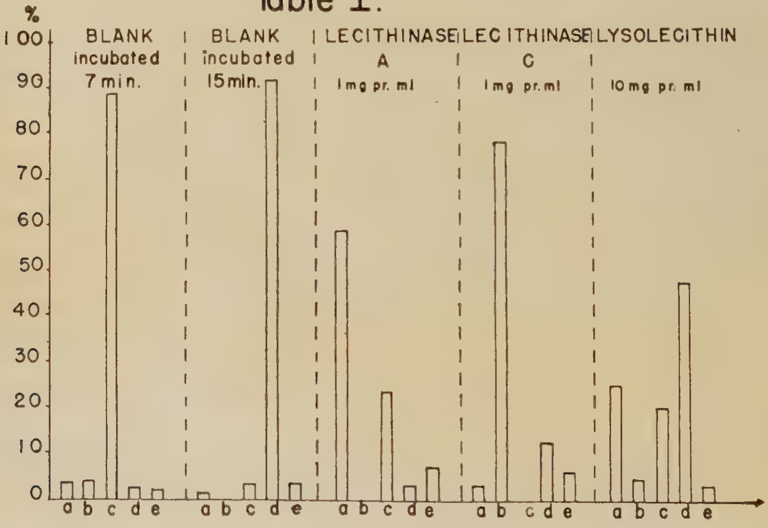
Trypsin. We used a crystalline preparation obtained from the pancreas of cattle according to the method of Northrop and Kunitz (5).

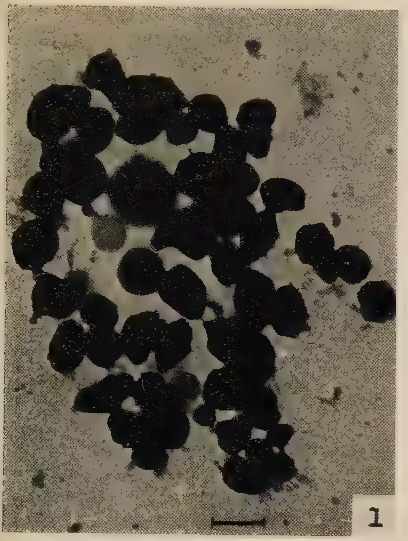
RESULTS AND DISCUSSION

Incubation time. The time of incubation had to be short enough not to produce morphological damage to the control sample. At the same time it had to be long enough to allow the hydrolytic enzymes to act. On incubation of the control for 15 minutes at 37°, definite morphological changes took place (see Fig. 2). These changes consisted mainly in enlargement of the particles, slight agglutination, and unsharpness of the peripheral part. After 7 minutes' incubation the damage to the control was not serious (see Fig. 1). *Since this incubation time was found to be satisfactory for the action of the hydrolytic enzymes on the mitochondria, it was used in all the experiments recorded below.*

The alterations caused by incubation for 7 minutes and for 15 minutes are characterized in Table I.

Table I.

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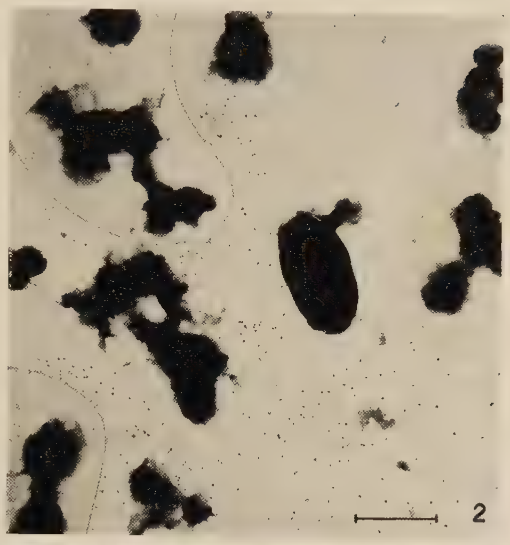
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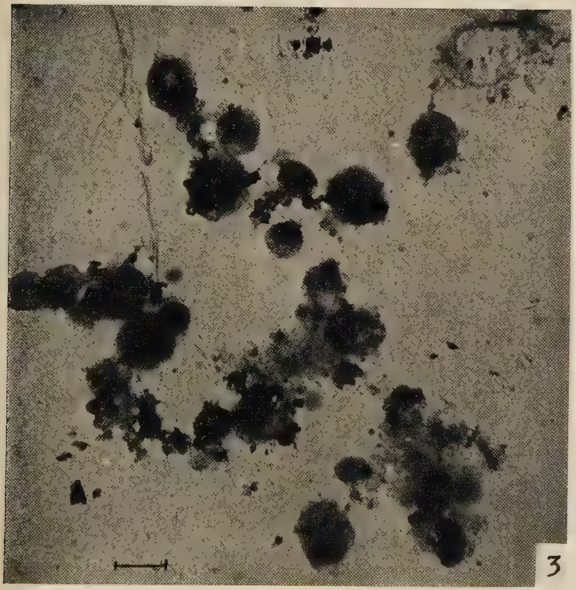
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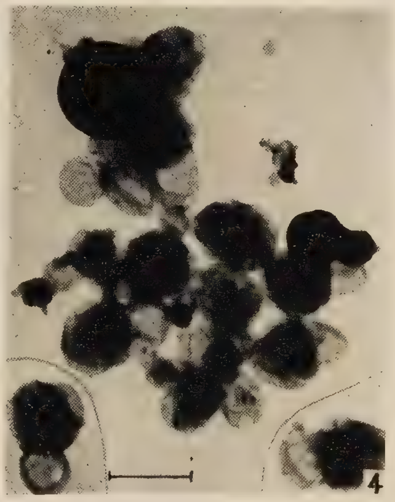
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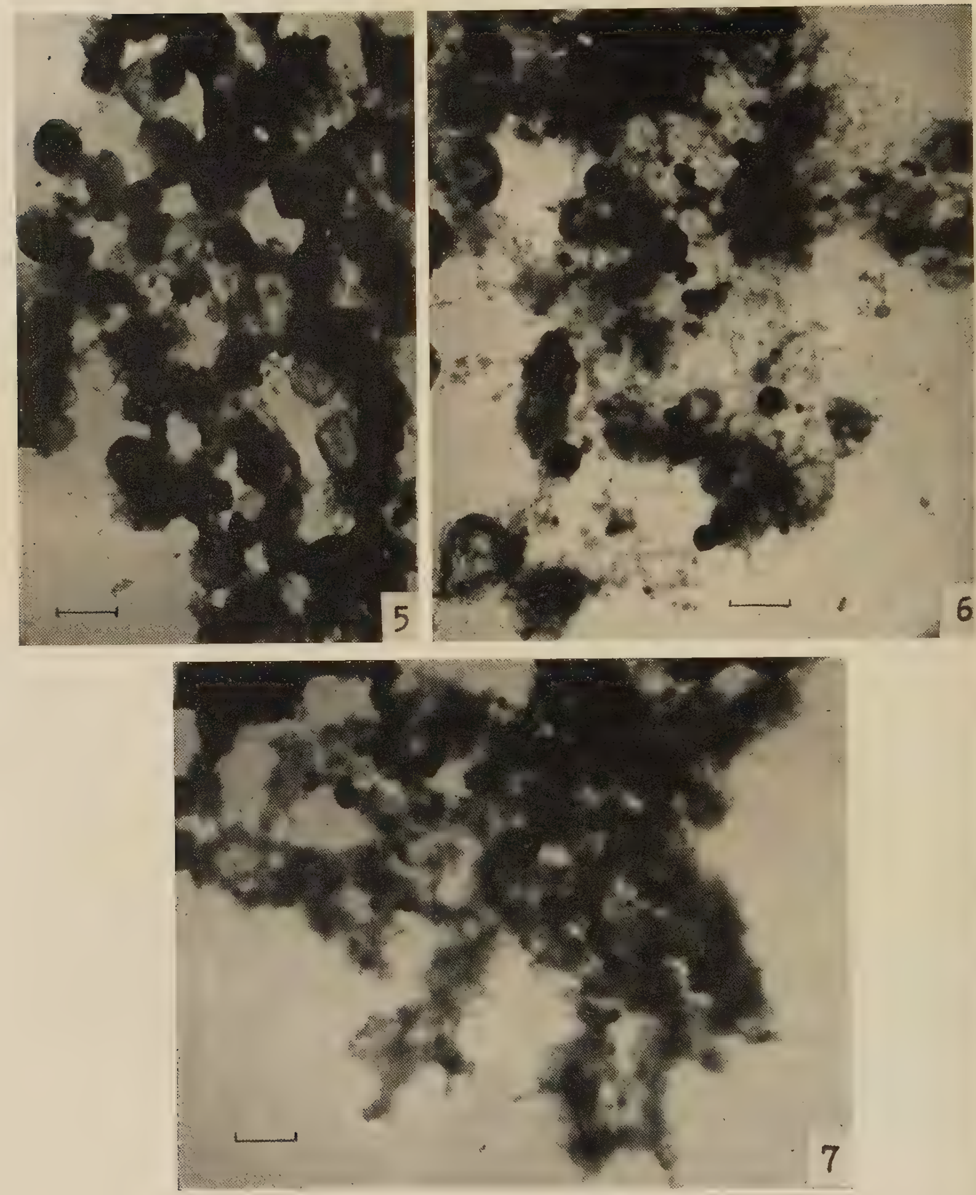
A 1 μ scale is drawn on all figures.

Fig. 1. Blank, incubated 7 minutes. Only small damage of mitochondria can be ascertained (8400 ×).

Fig. 2. Blank, incubated 15 minutes. Peripheral part of mitochondria becomes diffuse and loses material (13,200 ×).

Figs. 3 and 4. Mitochondria incubated with lecithinase A for 7 minutes. In Fig. 4, where the concentration of lecithinase A was 1 mg per ml, the mitochondrial membrane seems to be disrupted and partially dislocated at one side of the particle (13,200 ×). Slight changes of the peripheral part can be observed also in Fig. 3, where the concentration of lecithinase A was only 20 γ per ml (8400 ×).



Figs. 5, 6, and 7. Disintegration by lysophosphatides. Incubation time: 7 min. Fig. 5 shows agglutination and partial loss in electron density, caused by 0.1 mg per ml of lysophosphatides. The damage is more pronounced in Fig. 6 (1 mg per ml), and complete destruction is shown in Fig. 7 (10 mg per ml) (5600 ×).

The effect of lecithinase A. Typical morphological changes produced by lecithinase A are shown in Figs. 3 and 4 and characterized in Table I. The mitochondrial membranes seem to be partially disrupted and dislocated and form a bulge on one side of the particles. The density of the mitochondrial bodies is strongly reduced, particularly at the peripheries. The type of structural alteration appears to be the same with 20 γ and with 1 mg per ml of lecithinase A. From previous observations with the same sample of lecithinase A (6, 7) we know that incubation with 20 γ per ml will result in just a slight hydrolysis of the mitochondrial phospholipids, and that succinic dehydrogenase, cytochrome oxidase and DPNH oxidase are not appreciably inactivated under these conditions. However, the very sensitive factor located between cytochromes *b* and *c* in the succinoxidase system was found to be completely inactivated by this treatment. This is interesting, since 20 γ per ml of lecithinase A causes a dislocation of the mitochondrial membrane (see Fig. 3).

The effect of lysophosphatides. Incubation with lysophosphatides was found to inactivate the succinoxidase system. Using 10 mg per ml, the inactivation was complete under the conditions used in this study. With 0.1 mg per ml, less than 10 per cent destruction took place. It has been claimed (6, 7) that the inactivation of succinoxidase by lecithinase A could not be accounted for by a secondary effect of the lysophosphatides formed. It was therefore of interest to investigate whether the morphological effect of lysophosphatides was different from the one of lecithinase A. The morphological alterations caused by lysophosphatides are shown in Figs. 5, 6, and 7, and characterized in Table I. The extent of damage depends upon the concentration of lysophosphatides used (compare the figures). The alterations do not appear to be similar to the effects of lecithinase A.

The effect of lecithinase C and trypsin. The effect of lecithinase C and the effect of trypsin are characterized in Table I. The concentration of enzymes used were in both cases 1 mg per ml, and this was sufficient to inactivate the succinoxidase system. Neither lecithinase C nor trypsin appear to cause a morphological effect similar to lecithinase A.

The effect of different treatments on the morphology of isolated mitochondria.

Distribution of 300 counts of each sample.
a = detached membrane; b = light borders; c = normal; d = unsharp borders; e = light center.

SUMMARY

Rat liver mitochondria were incubated with lecithinase A, lysophosphatides, lecithinase C and trypsin, and the resulting alterations of the morphology of the particles were examined in the electron microscope. Lecithinase A, which is known to inactivate phospholipid-containing factors in the succinoxidase and the DPNH-oxidase systems, was found to cause a characteristic disruption and dislocation of the mitochondrial membrane; these alterations appeared to be different from the ones brought about by other reagents. Lysophosphatides were found both to disintegrate the mitochondria and to inactivate the succinoxidase system.

The authors wish to express their gratitude to Dr. K. G. Thorsson, Professor T. Caspersson and Professor H. Theorell for their interest in this work. One of us (A.P.N.) wishes to express his gratitude to A/S Freia Chokolade Fabriks arbeidsfond for ernæringsforskning for a personal grant.

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Experimental Cell Research, **6**, *459–478* (*1954*)

THE FERTILIZATION AND DEVELOPMENT OF THE SEA URCHIN EGG, *ARBACIA LIXULA*, UNDER THE INFLUENCE OF FRACTIONS OF EGG HOMOGENATE[1]

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Upon fertilization the egg of *Arbacia lixula* often forms a membrane that closely adheres to the egg surface. Below this membrane the hyaline layer appears. The fertilization membrane covers this layer as a thin, not very refractile gelatinous rim, see Fig. 15, where the hyaline layer constitutes the most conspicuous part of the non granular egg surface. From time to time batches of eggs were found in which the membrane elevated so that a perivitelline space appeared separating the fertilization membrane from the surface of the hyaline surface layer of the egg. In these cases the membrane is smooth and refractile. Even eggs with closely adhering membranes may be capable of normal development to the pluteus stage.

In previous work (4, 16) advantage was taken of the occurrence of the adhering membranes in order to test the promoting action of several substances on the membrane elevation. Among other things water extracts from the homologous eggs were found to cause the formation of elevated refractile membranes in eggs that in normal sea water formed closely adhering membranes upon fertilization.

This paper will give a preliminary report of studies on the action of different fractions of egg homogenate on the membrane elevation. In connection with this certain other phenomena will be touched upon, as the precipitation of the jelly coat under the action of "antifertilizin" (7, 18, 19). Some of the results have been briefly referred to earlier (4). In the quoted paper a descriptive nomenclature was proposed to designate the factors present in the extracts of sperm and eggs. In the following we shall be concerned mainly with two factors discernible by their effect.

I. The "Membrane elevating Factor" from the egg, abbreviated MbElF(E).
II. The "Jelly precipitating Factor" from the egg, abbreviated JePptF(E).

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[1] Dedicated to Professor Fritz Baltzer on the occasion of his 70th birthday.

The latter factor is synonymous with "antifertilizin" (7, 18, 19).

The designations used here refer to the main tests used for the demonstration of these factors. The aim of this research was in the first place to study the effect of the extracts on fertilization. In most cases, however, also the ensuing development was observed. Some data pertaining to the latter observations will be referred to.

METHODS

Unfertilized eggs of *Arbacia lixula* were washed several times with isotonic NaCl-solution and then sucked through a glass filter (French Pyrex E, pore size 100–120 μ). This was sufficient to break up the eggs. The granular components were spun down in a refrigerated centrifuge (8000 × g, 30 minutes). The granules were broken up by distilled water and then dialyzed against sea water. In the present experiments the supernatant was not submitted to any further fractionation. The crude supernatant served after dialysis against sea water as a source of the JePptF(E). Both the extracts from the granules and the dialyzed supernatant are used in different dilutions, see below. In some experiments the homogenization was carried out with eggs suspended in a mixture of NaCl and $CaCl_2$ present in about the same proportions as in sea water. As described by Hultin (5, 6) the granules break up to a certain extent in presence of calcium. This is obvious particularly by the release of pigment from the granules. Percentage figures are based on counts of 100–120 eggs each time.

RESULTS

The effect of MbElF(E) on the membrane elevation of the egg

Exp. I illustrates how the extract from the granular fraction of the eggs influences the membrane formation.

Exp. I. (*Naples, March 1952.*) Unfertilized eggs of *Arbacia lixula* were transferred to the following media:

a) 2 ml sea water;
b) 2 ml extract from the granular fraction diluted 1:10 with sea water (N-content: 40 μg per ml).

The eggs were inseminated after 30 minutes.

a) The eggs were fertilized, but the fertilization membranes were closely adhering to the egg surface;
b) The membranes were refractile and uniformly elevated from the egg surface.

The difference between the eggs in *a*) and *b*) became still more conspicuous when they were transferred to a hypertonic medium (1.6 ml sea water + 0.4 ml 2.5 N NaCl). The following observations were made.

a) The eggs assumed an angular form, see Fig. 1. No separation of the membrane occurred, even when the egg surface retracted.

b) Angular form more pronounced than in *a*, see Fig. 2. The separation of the membrane became only more evident than before the hypertonic treatment. The membrane remained distended.

It follows from Exp. I that in normal sea water the eggs formed a membrane which remained attached to the egg surface, whereas in the presence of the extract from the granular fraction the membrane separated uniformly from the egg surface.

A similar experiment as that described above was carried out with eggs deprived of their jelly coats subsequent to a brief treatment with acid sea water. This did not change the results. Closely adhering membranes were found in pure sea water, elevated membranes in sea water with extracts from the granular fraction. As reported in Exp. I the eggs were pretreated 30 minutes before insemination with the extract. The pretreatment was, however, not necessary. If eggs and spermatozoa were added simultaneously to the extract, the membrane elevation was as perfect as when the eggs had been pretreated with the extract. If, on the other hand, the eggs were fertilized some minutes before the addition of extract, no membrane elevation occurred.

The MbElF(E) not only improves the membrane formation, but also increases the percentage of fertilized eggs. Even if only 70–80 per cent of the eggs in sea water were fertilized, the eggs subjected to MbElF(E) gave 100 or close to 100 per cent fertilization. The cleavage was often found to be more regular and uniform in the presence of MbElF(E). The improving action of MbElF(E) is not species specific. Extract from the granular fraction of *Arbacia* eggs improved the membrane formation also in the sea urchins *Paracentrotus lividus, Psammechinus microtuberculatus* and *Sphaerechinus granularis*. Extract from the granular fraction of eggs of *Paracentrotus lividus* was tested on homologous eggs and on those of *Arbacia lixula, Sphaerechinus granularis*. In all cases there was a considerable improvement of the membrane elevation in eggs that in pure sea water exhibited closely adhering or irregularly elevated membranes. The percentage of fertilized eggs was also increased.

If the eggs were left in the (homologous) extract of the granular fraction the development often came to a stillstand in late blastula stage. The blastomeres were isolated from each other. This was probably due to a breakdown of the cement keeping the cells together. To bring the eggs to the pluteus stage it was necessary to transfer the eggs to normal sea water 5–6 hours after fertilization or to add sea water in order to dilute the extract to concentrations which were tolerated. In the pluteus stage the growth of the arms seems to be inhibited even in the presence of very dilute extracts.

If the eggs had been transferred in due time to normal sea water, a considerable improvement of the development was observed. This was less conspicuous in the spring of 1952 when the development of the *Arbacia* eggs was on the whole normal even if the membrane was closely adhering. In the spring of 1953 the development very often deviated from the normal course. As a consequence the improvement of the development was more striking.

Heat inactivation of MbElF(E)

The MbElF(E) from the granular fraction has proved to be thermolabile as the following experiment shows:

Exp. II (*Naples, March 1952*). Unfertilized eggs of *Arbacia lixula* were transferred to the following media:

a) 2 ml sea water;
b) 2 ml extract from granular fraction in sea water, diluted 1:10 with sea water (40 μg N per ml);
c) the same heated for 45 minutes at 32°C;
d) the same heated for 45 minutes at 45°C;
e) the same heated for 45 minutes at 55°C.

Ten to fifteen minutes after insemination the following observations were made:

a) 80 per cent of the eggs fertilized with closely adhering membranes;
b) 100 per cent of the eggs fertilized with well elevated refractile membranes;
c) result as under *b*);
d) almost 100 per cent fertilization; in most cases closely adhering membranes;
e) result identical with that found in *a*).

Similar experiments were carried out in the season 1953. The following experiment particularly emphasizes the changes occurring in the jelly coat.

Exp. III (*Naples, May 1953*). Unfertilized eggs of *Arbacia lixula* were transferred to the following media:

a) 2 ml sea water;
b) 2 ml extract from the granular fraction in sea water diluted 1:5 with sea water (150 μg N per ml);
c) as *b*), but the extract had been heated (before dilution) to 45°C for 30 minutes;
d) 2 ml 0.4 per cent serum albumin (640 μg N per ml);

After 10–15 minutes the following was recorded:

a) Closely adhering membrane with condensed jelly coat within which numerous spermatozoa were caught, see Fig. 3.

b) Refractile well elevated membranes; jelly coats strongly swollen. They seemed to offer no resistance to the spermatozoa, see Fig. 4.
c) Membranes closely adhering but with a tendency to local elevation. An indentation was present under the local elevation, see Fig. 5. The jelly coat was more swollen than in *a*), but considerably less than in *b*). The spermatozoa were caught in the jelly but more motile than in *a*).
d) Membrane elevated but to a lesser extent than in *b*). The membrane was less refractile than in *b*). The jelly coat was considerably more swollen than in *a*) and *c*), but less so than in *b*). Spermatozoa were often found in the jelly coat, see Fig. 6.

According to Exp. III there is a strict parallelism between the state of swelling of the jelly coat and the improvement of the membrane elevation. Polarisation microscopy was often used to get a more quantitative estimation of the properties of the membrane. The retardation of the membrane in the control with closely adhering membrane amounted to a value of 5–6 mμ, whereas in eggs subjected to the extract from the granular fraction the retardation of the membrane was 12–18 m μ. The thicker and more refractile the membrane appeared in ordinary light the higher the retardation. If, on the other hand, the eggs had been subjected to heated extract, the retardation was 5–6 mμ as in the control.

In general the cortical granules have merged with the vitelline membrane even in eggs with closely adhering fertilization membrane. Sometimes however, the eggs of the control formed granular membranes, i.e. the cortical granules were expelled without merging with the vitelline membrane (12), see Fig. 7. In the spring of 1953 it was often observed that the membrane was not continuous and gradually fell away from the egg surface in the form of flakes, although the membrane at its first appearance was closely adhering to the egg surface. The treatment with granular extract counteracted this tendency of the membrane to fall away as flakes.

On the other hand, a remarkable phenomenon occurred sometimes subsequent to fertilization in the presence of extract from the granular fraction. The membrane elevated as normally and was smooth and spherical. The increase in surface area continued, however, which resulted in a thinned out richly folded membrane. Fig. 8 shows an egg with a smooth membrane, Fig. 9 one with a folded membrane. Both eggs were from an experiment with extract from the granular fraction diluted 1:20 with sea water (110 μg N per ml). The retardation decreased during the thinning out of the membrane from 12–14 mμ to about 6 mμ. It seems rather probable that this decrease in retardation was due to a thinning out of the membrane. On direct inspection in ordinary light the folded membrane looks thinner and this impression may thus not be deceptive.

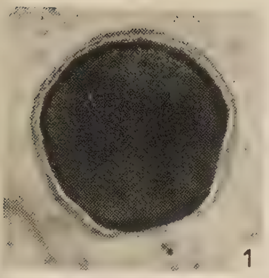
The normal increase in surface area when the membrane is elevated amounts to about 15 per cent in *Arbacia lixula*. The folding must cause a considerably greater increase in surface. The folded membrane was much less resistant than the smooth membrane. The former was often broken up at the first cell division possibly by the action of the telophasic elongation of the egg. Anyhow the thinned out membrane broke up and fell away after a varying time.

The granular fraction counteracts radialization

Particularly in the months of May and June 1953 the eggs of the control very rarely developed into typical plutei. In the majority of the experiments the eggs developed into radially symmetrical, slightly animalized larvae. The primary mesenchyme cells presented no bilateral arrangement and an increased number of triradiate skeleton spicules developed. The anterior part of the larva formed a ciliated lobe that to a varying extent was separated from the rest of the larva by a constriction. The hind part of the larva constituted a truncate anal field with the anal opening in the centre. The anal field is surrounded by a ciliary band which may assume a folded or lobed appearance, see further Gustafson and Sävhagen (1). In their paper good drawings of typical radialized larvae are reproduced. Figs. 10–13 represent a number of larvae from the controls of two different experiments (Naples, May 1953).

The larvae were fixed in dilute formalin (<1 per cent) in sea water and thereafter photographed. The larva of Fig. 10 belongs to the radially symmetrical type with endoderm of rather normal proportions. After fixation the anterior lobe seemed less separated from the rest of the body than in the living state. In Fig. 11 the shape is somewhat different. The truncate anal field is well pronounced. Figs. 12 and 13 represent two larvae with a more animalized character. Particularly in the larva represented in Fig. 13 there was an enlarged anterior region with a ciliary tuft. The endoderm was here more reduced than in the larva represented in Fig. 10. The extent of animalization could be estimated also by another character. No pigment cells appeared in animalized larvae, even if mesenchyme cells were present. Larvae of the type represented in Fig. 10 contained often many pigment cells but these could also be scarce in similar larvae. The more animalized larvae with enlarged ciliary tuft had few or no pigment cells.

It is characteristic of the radially symmetrical larvae that the animal-vegetal axis remains straight. As is well known the axis is bent in the bi-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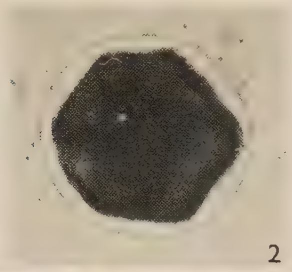
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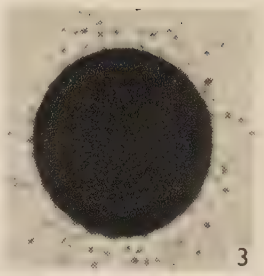
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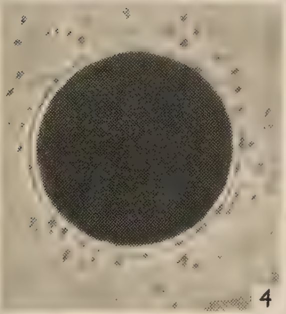
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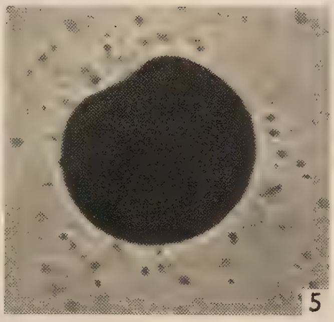
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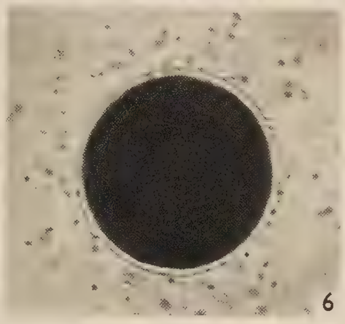
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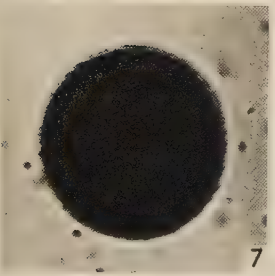
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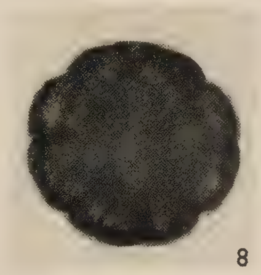
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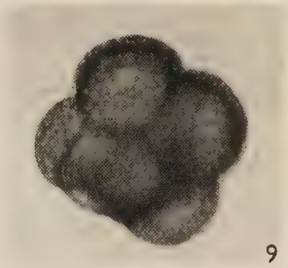
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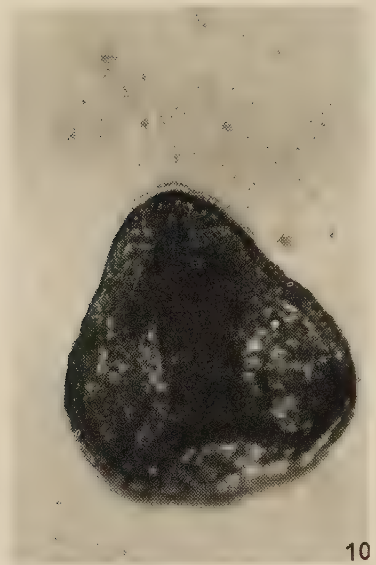
Fig. 1. Fertilized egg with closely adhering membrane, transferred to hypertonic medium, see Exp. I, *a*.
Fig. 2. Egg with membrane separated from the egg surface transferred to hypertonic medium, see Exp. I, *b*.
Fig. 3. Egg with closely adhering membrane and condensed jelly coat, see Exp. III, *a*.
Fig. 4. Egg with elevated membrane, jelly coat swollen, see Exp. III, *b*.
Fig. 5. Egg with closely adhering membrane except for a local elevation above an indentated region, see Exp. III, *c*.
Fig. 6. Egg with an elevated membrane, but less refractile than in egg on Fig. 5, see Exp. III, *d*.
Fig. 7. Egg with a closely adhering, granular membrane; jelly coat removed.
Fig. 8 and 9. Eggs fertilized in the presence of extract from the granular fraction of egg homogenate.
Fig. 8, egg with a smooth elevated membrane, Fig. 9, egg with membrane folded as a consequence of continued increase of surface.



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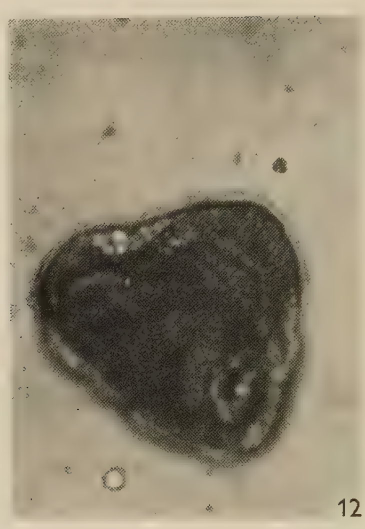

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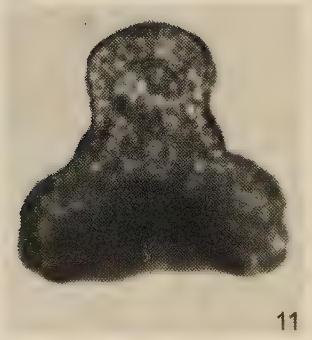

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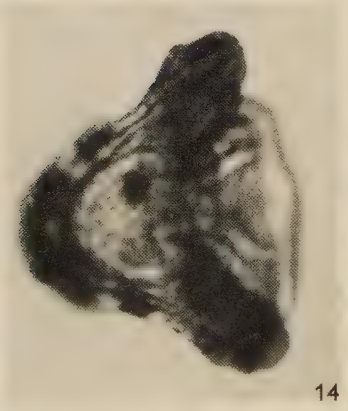

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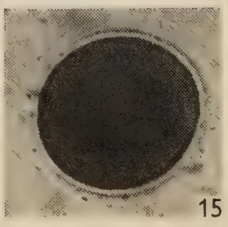

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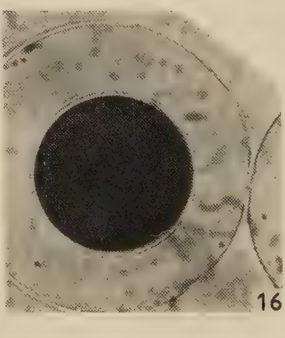

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lateral type. This is brought about mainly by a displacement of the animal pole in a ventral direction.

The eggs were transferred as in the experiments described above to extracts of the granular fraction diluted 1:10 with sea water. About six hours after fertilization the solutions were diluted about five times with pure sea water or the eggs were transferred to normal sea water. The treatment of the eggs with an extract from the granular fraction (N-content 80–90 μg per ml) removed the tendency to radialization. Normal bilateral plutei developed, see Fig. 14 (larva 42 hours after fertilization).

Heating of the extract to 45° or 60°C for 30 minutes made it inactive, so that all the eggs developed according to the radially symmetrical type. No detailed account will be given here on the variation which occurred with respect to the development type in larvae kept under the same conditions. The uniformity of development is great enough to warrant the general statements made above. A detailed study may in certain cases reveal intermediate types. The most common of this is one in which anal arms with normal skeleton supports were formed but the displacement of the animal region failed to occur. One may have all intermediates between such larvae and plutei with a certain hypertrophy of the animal part of the ciliary band that surrounds the oral field.

The presence of the intermediate types in question could in general be referred to an insufficient action of an extract added—due to a subthreshold concentration or to a decay of its activity, see for a pertinent example Exp. VI *c*. A certain tendency to secondary regulation toward a more bilateral organisation probably always prevails in the radially symmetrical larvae. It depends on a time factor whether this tendency will come to a visible expression or not.

Numerous observations made in course of the present experiments showed that the formation of closely adhering membranes does not necessarily result in a radially symmetrical larval type. Eggs with closely adhering membranes often developed into bilateral plutei as was observed, particularly in the "normal" season 1952.

The jelly precipitating factor

The supernatant after the sedimentation of the microscopic granules contains the jelly precipitating factor, JePptF(E) (antifertilizin, (7, 18, 19)). The factor in question precipitates the jelly coat of the egg. Certain details are given in Exp. IV.

Fig. 10–13. Radialized larvae of somewhat different types, see p. 464.
Fig. 14. Pluteus raised from eggs subjected to extract from the granular fraction, see p. 467.
Fig. 15. Fertilized egg with closely adhering membrane, see Exp. IV, *a*.
Fig. 16. Fertilized egg with jelly coat precipitated by addition of supernatant from centrifuged egg homogenate, see Exp. IV, *b*.

Exp. IV. (*Naples, March 1952*). Eggs of *Arbacia lixula* with jelly coat were transferred to:

a) 2 ml normal sea water;
b) 2 ml supernatant dialyzed against sea water diluted 1:10 with sea water (N content 185 per ml).
In *b*) a precipitation of the jelly coat occurred.

Both *a*) and *b*) were inseminated.

a) Only closely adhering fertilization membranes appeared, see Fig. 15.
b) The spermatozoa were able to penetrate the precipitated jelly coat and to fertilize the eggs. Almost invariably a refractile fertilization membrane was elevated, see Fig. 16.

In cases like that represented in Fig. 16 not only the surface of the jelly coat has precipitated; also the interior offered a resistance to the penetrating spermatozoa. It is seen on Fig. 16 that most of the spermatozoa were present in the peripheral part of the jelly coat. Conversely in the control egg of Fig. 15 all the spermatozoa have penetrated to the egg surface. If the precipitation was strong enough no spermatozoa were able to reach the egg surface; some of them penetrated into the interior of the jelly coat but remained here without reaching the egg. The high rigidity of the jelly coat evidently prevents their penetration to the egg surface. In supernatants diluted 1:10 usually almost 100 per cent fertilization occurred.

When eggs deprived of their jelly coats were transferred to the same solution, *no membrane elevation resulted* although almost 100 per cent of the eggs became fertilized. It is thus obvious that JePptF(E) does not in itself bring about a membrane elevation. This occurred only as a consequence of an interaction between the JePptF or some other factor present in the supernatant and the jelly coat.

Addition of 0.1–0.2 per cent serum albumin did not interfere with the precipitation of the jelly coat. Even at dilutions of the JePptF that no longer gave a visible precipitation of the jelly coat improvement of the membrane elevation still prevailed. Heating to 57° C for 45 minutes removed the jelly precipitating effect but the effect on the membrane elevation was not destroyed. Tenfold diluted supernatant was boiled for 20 minutes. As was to be expected this treatment removed any precipitating effect, but the membrane elevating action still remained.

The closely adhering membranes of jelly free eggs gradually changed their appearance if they remained in contact with the diluted unheated supernatant. The membranes became apparently very refractile, but did not elevate from the egg surface. One got the impression that the membranes had undergone a kind of tanning comparable to the toughening which is known to occur in the normal elevated membrane.

The effect of hypertonic treatment on the unfertilized egg in the presence of egg extract

A typical experiment pertaining to this topic is described below:

Exp. V (*Naples, May 1953*). Unfertilized eggs of *Arbacia lixula* were transferred to the following media:

a) 1 ml sea water;
b) 1 ml extract from granular fraction diluted 1:10 in sea water (110 μg N per ml);
c) 1 ml supernatant diluted 1:10 in sea water (93 μg N per ml).

After 30 minutes *a*)–*c*) were made hypertonic by addition of 0.25 ml 2.5 N NaCl. In *b*) the suspended eggs sank more rapidly than in *a*) indicating that the equilibrium volume was attained earlier in the former.

After 8–14 minutes the following observations were made:

	No activation	*Adhering membranes*	*Elevated membranes*
a)	100 per cent	—	—
b)	5 » »	22 per cent	73 per cent
c)	34 » »	64 » »	2 » »

Already 8 minutes after immersion in the hypertonic medium a portion of the eggs in *a*)–*c*) had been transferred to normal sea water (a^1–c^1); 45 minutes later a^1–c^1 were examined. The following observations were made:

a^1) No activation but in about 35 per cent of the eggs a seam is visible on the surface of the egg.
b^1) 95–100 per cent activation with a typical hyaline layer on the egg surface. The elevated membrane present before transfer to normal sea water had dissolved.
c^1) 60–70 per cent of the eggs activated, membrane present but closely adhering to the egg surface. No visible precipitation of the jelly coat.

After 180 minutes the eggs were examined again:

a^1) A certain activation (10–15 per cent of the eggs) was observed. Although the membranes were closely adhering to the surface they had dissolved to a varying extent. Flakes of the granular membranes were detached from the eggs.
b^1) No segmentation; monaster formation.
c^1) Adhering membrane still intact. No segmentation; small pigment-free exovates on the surface of the egg.

From these observations follows that the extract from the granular fraction enhances the activation of the egg by a hypertonic solution. Furthermore a membrane elevation occurs but the membrane is brittle and falls away in connection with the transfer to the normal sea water. This is reminiscent of what was described above, p. 463 and Fig. 9. However, a folding of the membrane was not observed in the hypertonic medium. Both in fertilization and artificial activation MbElF(E) thus favors the membrane elevation but not the membrane toughening. Possibly it counteracts a simultaneously operating membrane toughening effect. This is in keeping with the effect of the extract from the granular fraction on the cement substance keeping the blastomeres together. The supernatant, on the other hand, exerts a conserving effect on the membrane (see Exp. V*c* and c^1). The maintenance of the membrane in this case was not due to the fact that they adhered closely to the egg surface. This was also the case in the eggs not pretreated with extracts (see Exp. V*a* and a^1). But here the membrane fell away in flakes. From this it is apparent that the supernatant contains a membrane toughening factor.

The effect of Ca on the distribution of jelly precipitating and membrane elevating factor

The experiments referred to in the previous sections were carried out with homogenates prepared in NaCl-solution. In this section it will be shown that somewhat different results were obtained if the homogenization was carried out in a mixture of NaCl + $CaCl_2$. As previously the granular fraction was dissolved in distilled water and dialyzed against sea water. The supernatant was also dialyzed against sea water. The supernatant now contained more of the membrane elevating factor, whereas the granular fraction contained less of the factor as compared with the homogenates prepared in the presence of NaCl alone, see Exp. VI.

In the description of the experiments the following designations will be used:

Granular fraction of homogenates prepared with NaCl: NaCl granular extr.
The same prepared with Na + $CaCl_2$: (NaCl + $CaCl_2$) granular extr.

The corresponding designations for the supernatants will be NaCl Supernatant and (NaCl + $CaCl_2$) Supernatant.

Exp. VI (*Naples, May 1953*). Eggs were transferred to the following media:

a) 1 ml sea water;
b) 1 ml NaCl granular extr. in sea water, 1:10 (87 μg N per ml);
c) 1 ml (NaCl + $CaCl_2$) granular extr. in sea water, 1:10 (66 μg N per ml);
d) 1 ml serum albumin 0.2 per cent (320 μg N per ml).

After 10 minutes the eggs were inseminated. The following observations were made:

a) The fertilization membrane adhered to the egg surface or was dissolved. Under the latter conditions the cortical granules were exuded, but never contributed to the formation of a continuous membrane.
b) 56 per cent of the eggs presented a well elevated refractile membrane. The remainder were fertilized eggs with dissolved membrane, cortical granules were released in the surface of the egg.
c) 45 per cent of the eggs had elevated a membrane that was thinner and less refractile than in *b*). In the course of cleavage the membrane was more often broken up in *c*) than in *b*). The eggs without membrane as in *b*).
d) The eggs resembled those in *a*).

The eggs developed in *a*)–*d*); in early blastula stage pure sea water was added to the cultures so as to decrease the concentration of the extracts.

The following gives a survey of the state of development after about 40 hours.

a) Radially symmetrical larvae with lobelike animal part. The hind end truncate surrounded by a ciliary band, cf. Fig. 11. Endoderm underdeveloped.
b) Bilateral plutei with well developed skeleton and normal endoderm. The animal part with oral arms somewhat underdeveloped. In some cases a stomodaeum had appeared. Endoderm normal.
c) The radially symmetrical type dominated but in many cases there was an elongation of the apical part of the larva that in other respects had the same type as radial, to some extent animalized larvae. Intermediate types between the radial and bilateral larvae arose in this way. Finally some single plutei of type *b*) were observed.
d) The larvae resembled those in *c*), but no plutei were observed.

The control *a*) of the above experiment thus exhibited radialized larvae as almost invariably occurred during the season May-June 1953. The addition of extract from the granular fraction proved capable of switching the development into the bilaterally symmetrical course.

As follows from Exp. III albumin may cause an elevation of the fertilization membrane of *Arbacia lixula* but is less efficient than the extracts from the granular fraction both in causing membrane elevation and normal development. The underdevelopment of the animal part in eggs pretreated with extract from the granular fraction (Exp. VI *b*) was due to the fact that the extract was not completely washed away. Even if present in traces the extract may cause a reduction of the most animal parts including the oral arms. If the extracts were carefully washed away no such underdevelopment occurred.

The supernatants obtained after spinning down the granules used in Exp. VI were also tested. The results are given below.

Exp. VII (*Naples, May 1953*). Unfertilized eggs of the same material as used in Exp. VI were transferred to the following media:

a) 1 ml sea water;
b) 1 ml NaCl supernatant in sea water (184 μg N per ml);
c) 1 ml ($NaCl + CaCl_2$) supernatant in sea water (201 μg N per ml);

After 20 minutes *a*)–*c*) were examined:

a) many eggs have lost their jelly coats;
b) precipitation of the jelly coats, macroscopically visible agglutination;
c) very weak precipitation of the jelly coat, no visible agglutination.

In a parallel series a^1)–c^1) the eggs had been pretreated with acid sea water in order to remove the jelly coats. Usually the treatment did not give a yield of 100 per cent, i.e. besides a majority of eggs devoid of jelly there still was a number of eggs surrounded by a jelly coat. After a pretreatment for 40 minutes with the media, the eggs were inseminated.

The following observations were made 15 minutes after insemination:

a^1) Fertilization >99 per cent, membrane always closely adhering but in many cases dissolved; in the latter case cortical granules were seen to be expelled upon fertilization.
b^1) Fertilization as in a^1), membrane closely adhering with exception for eggs with jelly coats; usually they fertilized despite the precipitation of the jelly coat and presented a pronounced elevation of the membrane within the jelly.
c^1) Fertilization as above; membrane always well elevated with exception for a smaller number of cases where the membrane was dissolved with expulsion of cortical granules.

After about 40 hours a survey of the development of the larvae was made:

a^1) Larvae of radially symmetrical type;
b^1) The same;
c^1) The tendency for bilateral symmetry more pronounced. No oral lobes were observed; the larvae had pluteus shape but were often without arms. A rudimentary stomodaeum sometimes appeared.

It follows from Exp. VII that the homogenization in $NaCl + CaCl_2$ lowers the JePptF-effect of the supernatant as compared with that from homogenates prepared with NaCl. On the other hand, it turned out that the development was more normal after treatment of the eggs with ($NaCl + CaCl_2$) than with NaCl supernatant.

DISCUSSION

The data presented above must be regarded only as a first approach to studying the effect of different fractions of the homogenized eggs on the fertilization and development. The granular fraction is not uniform; further work should involve a fractionation of the granules. Moreover it may be questioned whether the MbElF-effect of the granular egg fraction is a specific one. It is known (14) that proteins and amino acids improve the membrane formation. It seems sufficient to assume that the extracts from the granular fraction exert their action in virtue of their protein nature. The extracts are active, however, at a much lower concentration expressed as nitrogen per ml than serum albumin as follows for example from Exp. VI, p. 470. In Exp. III, p. 462, a concentration of serum albumin twice that used in Exp. VI proved to be active, but the result was more pronounced upon addition of the extracts, see Figs. 5 and 6. More systematic studies are no doubt needed. The thermolability of the MbElF(E) is remarkable, and points also to something more specific than a general protein effect. Anyhow, the data show that the granular fraction contains a substance which even in rather low concentrations is able to favour the membrane elevation The mechanism of this action will not be further discussed here, but it may be pointed to the remarkable further increase in surface area of the membrane which sometimes occurs in the presence of extract from the granular fraction, see p. 463 and Figs. 8 and 9.

Our present data cannot decide whether the action of the extract is on the eggs, on the spermatozoa or on both. The last possibility seems the most likely. Eggs which are refractory to membrane elevation do not improve even when quite fresh sperm is used. Moreover, it was shown that extract from the granular fraction markedly changes the physical properties of the egg surface, see (21).

The data presented in Exp. VI and VII, p. 470–472 show clearly that homogenization in the presence of calcium (concentration corresponding to that prevailing in sea water) opens the granules so that the content of MbElF decreases, whereas it increases in the supernatant. This is in keeping with the results obtained by Hultin (5, 6) who demonstrated that addition of calcium to a suspension of the granular fraction causes a sort of breakdown of the granules. The results of Hultin may point to a role of calcium in the fertilization. The importance of calcium has been emphasized by several authors, particularly by Heilbrunn (2, 3); for a brief survey of this question, see (12, p. 295). Possibly a release of calcium may "open" granular components so as to establish an interaction between the interior of the granules and the cytoplasmic ground substance. The ease at which the granules are opened plays probably a role for the rate of the cortical changes occurring upon fertilization. In other words it can be supposed that the release of MbElF from certain granules of the egg cell plays a role also in the normal fertilization process.

The jelly precipitating factor is present in the supernatant of the egg homogenates after the spinning down of the microscopic granules. As has been reported briefly elsewhere (4) a further fractionation of the highly heterogenous supernatant has already been carried out. Here, however, only the properties of the non fractionated supernatant will be considered. The supernatant does not promote the membrane elevation of the jellyless eggs — even if the protein concentration on basis of nitrogen content is higher than in the granular fraction. By an interaction of the supernatant with the jelly coat a membrane elevating factor is brought into operation. Pending further experiments a discussion of the nature of this interaction will be postponed.

As has been briefly described elsewhere, the jelly precipitating factor (4) present in sperm, JePptF(Sp), exerts the same kind of interaction with the jelly. It thus promotes the membrane elevation in eggs surrounded by their jelly coat.

A basic protein like clupein also precipitates the jelly coat (14) but it was not found to exert a similar interaction with the jelly as extracts with the JePptF(E) or (Sp). If the spermatozoa were able to penetrate the jelly coat precipitated with clupein an adhering membrane appeared; the cortical granules were attached to the membrane without merging with this, see (14). If extract from the granular fraction (40 μg N per ml) was added to relatively low concentrations of clupein (e.g. 0.005 per cent in sea water) an elevation of the membrane often occurred. If eggs were fertilized in the presence of this concentration of clupein alone a closely adhering granular membrane appeared. In higher concentrations of clupein (e.g. 0.02 per cent) its action predominated, e.g. the membrane adhered to the egg surface even if extract from the granular fraction was added.

Monroy and Runnström (9, 10) demonstrated that in *Arbacia punctulata* the membrane may change its properties under the effect of some substance diffusing from the egg. Its release was favored when the eggs were fertilized in the presence of iodosobenzoic acid. The membrane became apparently thicker or more refractile. The retardation increased which may indicate an increased thickness or possibly may be due to an increased orderliness of structure. Motomura (11) also presented evidence for the view that a "membrane toughening factor" is present in the egg. Motomura discussed the possibility that the toughening factor is identical with JePptF(E). This view gets some further support by an observation reported above (p. 468–469). The apparent thickness of the adhering membranes increases strongly in eggs which remain in contact with the supernatant of the egg homogenates obtained after the spinning down of the microscopic granules.

A similar change occurs if the eggs with adhering membranes are subjected to strong dilution of the medium. Also an elevation of the membrane is observed under these conditions. It is evident that substances which influence the membrane are released from the egg surface. In some cases it could be observed upon dilution of the medium how the change of the membrane started at points adjacent to more strongly injured regions of the egg surface.

In this context one may remember Exp. V, p. 469 in which the eggs were subjected to a hypertonic solution. In the presence of extract from the granular fraction, a membrane elevation took place, but the membrane was dissolved when the eggs were transferred to normal sea water. If the eggs had been treated with the hypertonic solution in the presence of the corresponding supernatant, only closely adhering membranes appeared, but these did not dissolve when the eggs were brought to normal sea water, although a dissolution of adhering membranes was often observed even in normal sea water. This experiment seems to favor the view that the supernatant contains some membrane toughening factor, possibly identical with JePptF.

Some other phenomena may be mentioned here which point to the diffusion of active substances from the eggs into the medium. Unfertilized eggs subjected to a hypertonic medium gradually smoothen (13, 20, 21). It has been observed that the smoothening starts in smaller groups of eggs and then spreads like a contamination to encompass an increasing number of eggs. The smoothening seems to be accompanied by the release of a substance. The threshold for the smoothening and subsequent release of substance may be different in different eggs. The eggs with lower threshold may exert an inducing effect mediated by the diffusing substance. The substance in question may be identical with MbElF(E) which also smoothens the egg in hypertonic medium. According to Lundblad (8) one of the proteolytic enzymes with pH-optimum at 7.3 present in the egg diffuses out into the medium when the eggs are exposed to a hypotonic medium. The enzyme probably has a superficial location and may be one of the factors involved in the membrane formation.

In inseminated jelly-free eggs there is often a "neighbour effect". Where two or three adjacent eggs touch each other the membrane is often better elevated and more refractile than in the parts of the egg which are more removed from each other. Here again the observations point to an interaction between the adjacent parts. Both MbElF and a toughening factor seem to be released. Apparently an induction through diffusion is operating also in these cases.

The observation on the development of the eggs show clearly that the extract from the granular fraction counteract the intrinsic tendency to radialization or slight animalization which usually characterized the *Arbacia* eggs during a certain period of the breeding season 1953 (see Figs. 10–13 as contrasted with Fig. 14).

The thermal inactivation of the anti-radialization effect coincided with that of MbElF(E), see p. 467. An identity is thus possible. The anti-radialization effect is probably not merely the consequence of the membrane elevation, as normal larvae may arise from eggs with closely adhering membranes, see p. 467. The supernatant containing the JePptF(E) does not seem to have an anti-radialization effect.

Many more experiments about the anti-radialization effect are necessary. Nevertheless it seems possible that a factor operating in the activation process also plays a role in the further development. This suggestion is in keeping with the results and conclusion reached by Shaver (17) for the development of the frog. Certain granules from the gastrula stage have a cleavage initiating effect when injected into unfertilized eggs. These granules, on the other hand, arise in the stage when the induction processes begin to operate. In the sea urchin development an interaction between granular components of microscopic dimensions and surrounding submicroscopic components of fibrous or globular shape may contribute to the regulation of the balance between ventral and dorsal differentiation.

SUMMARY

Unfertilized eggs of *Arbacia lixula* were homogenized in the presence of isotonic NaCl solution. The microscopic granules were spun down by centrifugation for 30 minutes at 8 000 g.

The granular fraction was dissolved in distilled water and dialyzed against sea water. The supernatant was also dialyzed against sea water. Before being tested both the extract from the granular fraction (I) and the supernatant (II) have been diluted with sea water, usually 1:10.

I contains a membrane elevating factor, MbElF(E); II contains antifertilizin or according to the terminology adopted above the jelly precipitating factor, JePptF(E). (E) indicates that the factors are obtained from the eggs. Upon insemination of the *Arbacia* eggs in normal sea water the eggs are fertilized but in most batches of eggs the membrane adheres closely to the egg surface and has not the optical properties of the normal membrane. In the presence of MbElF(E) a membrane elevation occurs. The membrane becomes more refractile and more solidified. The retardation is higher than in

the adherent membranes. Heating of the extract from the granular fraction to 45° or 60° C for 30 minutes decreases or abolishes the MbElF-effect.

Eggs with jelly precipitated under the action of JePptF(E) can be fertilized and they exhibit an elevated refractile fertilization membrane. Even the heated II has this effect, although no precipitation of the jelly coat occurs. In eggs deprived of their jelly coats an adherent membrane is formed upon fertilization in the presence of II. JePptF thus causes no elevation of the membrane in absence of the jelly coat. Some kind of interaction between JePptF and the jelly coat is thus necessary to induce the membrane elevation. If jellyless fertilized eggs remain in contact with II a sort of tanning of the membrane occurs which remains adherent to egg surface. This observation speaks in favour of the presence of a membrane toughening factor in II.

MbElF(E) favours the membrane elevation also when unfertilized eggs are subjected to a hypertonic solution.

In certain experiments the homogenization was carried out in a mixture of NaCl and $CaCl_2$ (concentrations as in sea water). It was evident from the results that in the presence of calcium MbElF(E) is partly released from the granules. After previous homogenization in the presence of Ca the MbElF-action is thus also found in II.

Under a certain season the control almost invariably gave radially symmetrical or slightly animalized larvae. This tendency was counteracted when the eggs were fertilized in and reared 5 6 hours after fertilization in sea water containing extract from the granular fraction. Like MbElF(E) the anti-radialization factor was inactivated by warming to 45° or 60° C for 30 minutes. These results suggest that a release of substances from the microscopic granules in the egg may play a role for the balance between ventral and dorsal tendencies in the early sea urchin development.

This work was carried out at the Stazione Zoologica at Naples. We express our sincere thanks to Professor R. Dohrn for his kind interest and generous help. Our thanks are also due to the Natural Sciences Research Council for financial support. One of us (E. W.) received also grants from "Hierta-Retzius Stipendiefond" and from "C. F. Liljevalch J:rs Stipendiefond".

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A METHOD OF DETERMINING THE FERTILIZATION RATE IN SEA URCHINS

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At the beginning of the spawning season of sea urchins a period of refractoriness with respect to fertilization has often been observed. This phenomenon has particularly been studied in *Psammechinus miliaris* from the Swedish west coast. These writers have observed the same condition in a number of species in Naples, Roscoff, and Tromsö. Runnström and co-workers ascribed this state of refractoriness mainly to a cytoplasmic underripeness of the egg (for previous works cf. (6)). Runnström *et al.* (8) found that exposure to rabbit or sheep serum or to glycine and several other amino acids caused a considerable improvement in fertilization (cf. (12)). Later, Runnström and Kriszat (7) showed that treatment with sodium periodate also neutralizes this underripeness. Glycine, periodate and the other agents mentioned cause a remarkable swelling of the jelly layer. However, this is not the whole explanation of their improving influence, since it has been demonstrated that eggs deprived of their jelly show improved fertilization (6).

It has also been shown (9, 11) that albumin and amino acids prolong the fertilizing capacity of the sperm; thus evidently these substances affect both gametes.

In an attempt to study the effect of different agents on the fertilization of the sea urchin egg it seemed desirable not only to indicate the percentage of fertilized eggs or the percentage of eggs with the fertilization membrane elevated. In addition it would be valuable to have a measure of the rate of fertilization in a population of eggs. For this purpose a method is needed which allows a quantitative evaluation of the number of eggs fertilized different intervals of time after the insemination.

Rothschild and Swann (4) already described an experimental procedure by means of which a quantitative study of fertilization is possible. This procedure, however, is not easy to handle and not suitable in elaborate serial experiments.

Harding and Harding (2) described a method which is more convenient.

The fertilization was inhibited after different intervals by the addition of heparin. However, there is a definite difference in the inhibiting capacity of heparin preparations from various sources and this excludes a general application of the method.

The writers first tried crude preparations of fucoidin. It is known that these contain an inhibitor of fertilization (1). However, it was observed that a certain percentage of the eggs was fertilized in the course of 20–60 minutes even in the presence of the inhibiting factor. Therefore, this factor was not considered to be suitable for our purpose. In order to get an evaluation of the rate of fertilization it is essential to eliminate the sperm momentarily without injuring the eggs. This paper presents a convenient method of determining the rate of fertilization and demonstrates the use of this method in the study of the problem of underripeness and its removal by serum albumin, glycine and sodium periodate.

METHODS

To eliminate the spermatozoa, sodium-lauryl-sulfonate, a detergent, was tried and found very suitable. This substance in concentrations of 0.0005–0.0007 per cent immediately stopped the motility of the spermatozoa. There was no visible injury to the unfertilized eggs even after several hours of treatment. The development of the fertilized eggs was normal.

The animals were opened and the ovaries put into a beaker containing sea water. The contents of the beaker was passed through fine silk cloth and the eggs were washed by sedimentation.

Aliquots of the eggs were treated for 15 minutes with albumin (crystalline albumin from Armour Co.), glycine (Merck, Darmstadt) and sodium periodate (G. F. Smith Co.) respectively and then washed twice with sea water (e.g. 10 ml of sedimented eggs were diluted to a volume of 250 ml). The egg suspension was inseminated, and after different intervals (3, 5, 10, 20 ... 180 seconds) 10 ml of the suspension was poured into beakers containing an appropriate quantity of sodium-lauryl-sulfonate. In all the experiments, the number of sperm was 2.6×10^6/ml. The egg suspension and the beakers were kept in motion to ensure rapid mixing of the eggs, sperm and sodium-lauryl-sulfonate. The control was treated in exactly the same manner, except that the treatment with albumin etc. was omitted. The eggs were then allowed to cleave in order to facilitate the determination of fertilized eggs. About 250 eggs were counted in each beaker.

RESULTS

The experiments with albumin were carried out at the Stazione Zoologica, Naples, using *Paracentrotus lividus* as material. The concentrations of albu-

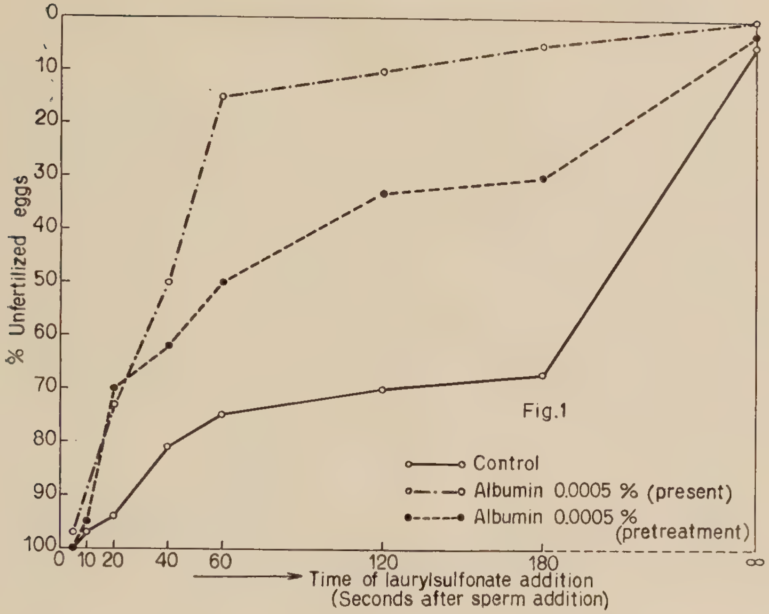

% Unfertilized eggs
0
10
20
30
40
50
60
70
80
90
100
0
10
20
40
60
120
180
∞
Time of laurylsulfonate addition
(Seconds after sperm addition)
Control
Albumin 0.0005 % (present)
Albumin 0.0005 % (pretreatment)

Fig.1

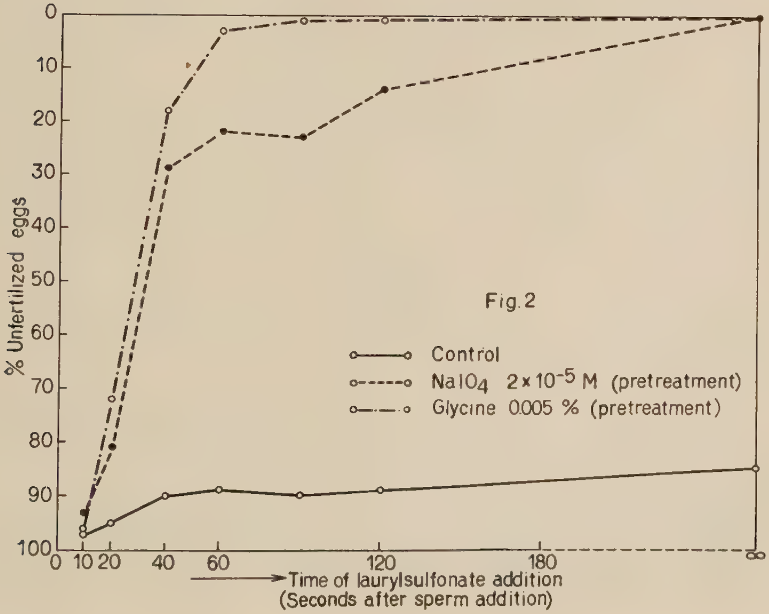
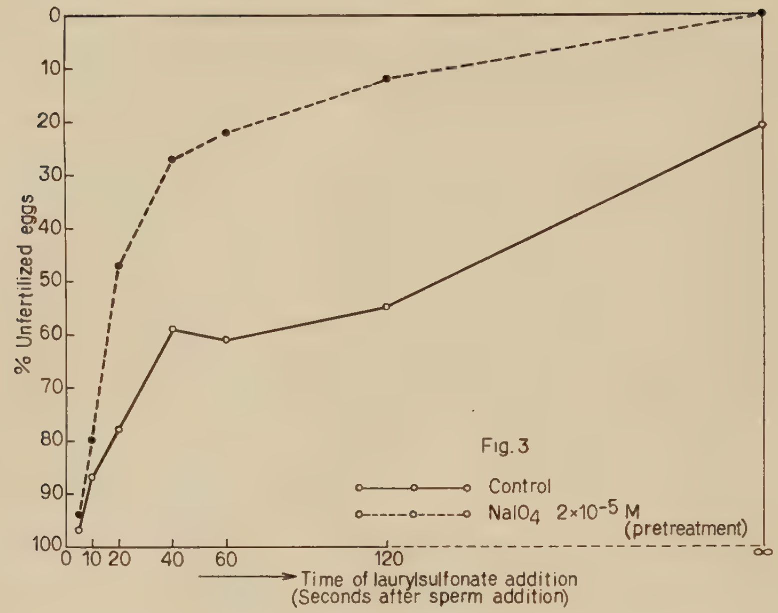

% Unfertilized eggs
0
10
20
30
40
50
60
70
80
90
100
0
10
20
40
60
120
180
∞
Time of laurylsulfonate addition
(Seconds after sperm addition)
Control
NaIO$_4$ 2×10^{-5} M (pretreatment)
Glycine 0.005 % (pretreatment)

Fig. 2



min employed ranged from 0.00005 to 0.2 per cent. The highest concentration gave about 95 per cent fertilization in 5 seconds, so there seemed to be no point in using higher concentrations. In the lowest concentration, however, there was a very weak promoting action if any. Fig. 1 illustrates an experiment in which the concentration of albumin was 0.0005 per cent. The egg suspension was pretreated for 15 minutes and then divided into two equal aliquots, one of which was immediately inseminated and the other was washed before insemination. The difference between the pretreated eggs and the control was distinct. There was also a significant difference between the pretreated eggs and the eggs fertilized in the presence of albumin. This latter difference indicates the improvement of the spermatozoa caused by the presence of albumin.

The experiments with glycine and sodium periodate were carried out in Naples on *Paracentrotus lividus* and on *Echinocardium cordatum* at the Kristineberg Zoological Station on the Swedish west coast. In these experiments also, several concentrations of the agents were tried. As a rule the time of treatment was 15 minutes and the concentration of glycine 0.05 per cent and that of $NaIO_4$ 2×10^{-5} molar. Two experiments are shown in Figs. 2 and 3.

DISCUSSION

As shown by Wicklund (11), albumin has an improving influence on the vitality and fertilizing capacity of the spermatozoa. The observations reported by Runnström (5) and the experiments where glycine was present at fertilization (12) undoubtedly indicate that the condition of the eggs has become improved and that the underripeness has been eliminated. Runnström and his school have shown that there is an improving action of proteins, amino acids and sodium periodate on the condition of eggs and sperm.

Tyler (10) has denied that there are underripe eggs and has furthermore claimed that treatment of the sperm alone can overcome low fertilizability. He has also stated that pretreatment of the eggs "gives no marked improvement in fertilization". Tyler's own results (10), however, seem not to justify this conclusion. As seen from his Table IV (10) the improvement of the versene-pretreated eggs is relatively more pronounced than that of the eggs fertilized in the presence of versene. Untreated eggs show 30 per cent and eggs pretreated with the lowest concentration of versene 100 per cent fertilization. This indicates a striking improvement resulting from pretreatment of the eggs.

The present work demonstrates that fertilization is more rapid subsequent to pretreatment with albumin, glycine and $NaIO_4$. The number of fertilized eggs is also increased. In cases where untreated eggs already are mature, i.e. give 90–100 per cent fertilization, the rate of fertilization is increased following pretreatment. The objection is possible that albumin and glycine are absorbed by the jelly layer in spite of washing and thus possibly exert an indirect action on the spermatozoa. In the case of sodium periodate this objection cannot be valid since the breakdown of periodate is rapid (3).

SUMMARY

A method for estimating the rate of fertilization has been described.

The fertilization rate is increased by pretreating the eggs with albumin, glycine or sodium periodate. These substances facilitate fertilization in underripe sea-urchin eggs.

We are very much indebted to Professor John Runnström for his interest in this problem and for valuable discussions and criticism. Our thanks are also due to Dr. R. Dohrn, Naples and Dr. G. Gustafson, Kristineberg, for generous help and supply of material. This work was supported by the Swedish Natural Science Research Council.

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STUDY ON THE EFFECT OF ANTISERA ON UNFERTILIZED SEA URCHIN EGGS

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In three previous papers (1, 2, 3) the results of some serological experiments with extracts from lyophilized sea urchin gametes have been reported. A preliminary report will now be rendered on the influence of antisera upon living unfertilized sea urchin eggs.

MATERIAL AND METHODS

The experiments were carried out at the Zoological station at Naples, Italy, and the sea urchin eggs used were from the common Mediterranean species *Paracentrotus lividus*, *Arbacia lixula*, and *Psammechinus microtuberculatus*. 0.5 ml of a concentrated egg-suspension was pipetted into Boveri dishes. In general, eggs from only one female were used in each experiment. In some experiments isotonic sucrose solution was added to the dishes.

The antisera had been obtained by injecting suspensions of lyophilized egg homogenate into rabbits. In the case of the species *Paracentrotus lividus*, antisera had also been prepared against sperm, the dissolved jelly and the supernatant of egg-homogenate after 1 hour centrifugation at 65000 × g. The antisera, which were free from preservatives, were identical to those used in the previous investigations mentioned above (1, 2). Before use, the sera were dialyzed against sea water, and if necessary, the pH was adjusted to 7.8 by the addition of NaOH. In all experiments normal, dialyzed rabbit serum was used as a control. The sera were added to the egg-dishes in varying amounts, and the content of the dishes brought to a certain volume by the addition of sea water. The final concentration of the sera varied from 5–50 per cent in the different experiments. In some experiments, the serum treatment was interrupted after a certain time by centrifuging down the eggs with a hand-centrifuge and washing 3 times with sea-water.

RESULTS

Addition of a homologous antiserum to the eggs caused a coagulation of the jelly layer surrounding the unfertilized egg. With the exception of *A. lixula*, this coagulation occurred (or at least, was visible) only if the electrolyte-content of the medium was lower than that of sea water. Optimal conditions for coagulation were obtained by the addition of isotonic sucrose solution to a final ratio of 1:1. A few minutes after the addition of antiserum in sufficiently large amounts, a clearly visible ring-shaped precipitation appeared at the surface of the somewhat swollen jelly layer (Fig. 1). Although the swelling of the jelly can be regarded as a non-specific effect of the serum proteins on the jelly substance (cf. 5), the observed precipitation was species specific to a fairly high degree (Table I).

TABLE I

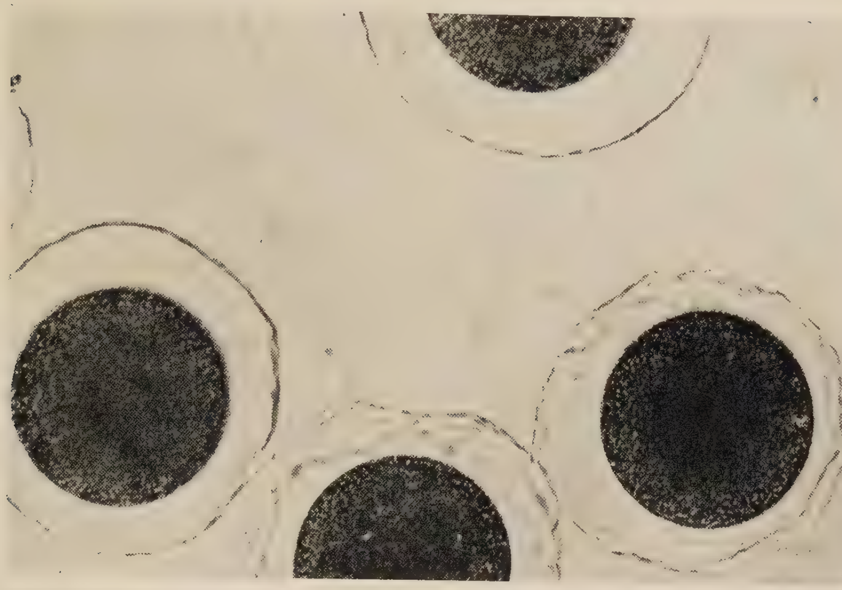
Effect of antisera on the jelly layer and on the cell surface.

Antisera for	Eggs from		
	Paracentrotus	*Psammechinus*	*Arbacia*
Paracentrotus	+ +	+	±
Psammechinus	±	+	0
Arbacia	0	0	+ +
	0	0	0

Table I represents a summary of the results of about 40 experiments in which optimal serum concentrations (about 30 per cent) were used (Stronger concentrations gave the same results). + +: visible effect on about 90 per cent of the eggs, +: visible effect on an intermediate number of eggs (in most cases 40–60 per cent), ±: visible effect on less than 10 per cent of the eggs, 0: no visible effect.

In experiments with homologous antisera, the lowest active concentrations were 8–10 per cent for all species, while heterologous antisera had to be added in larger amounts to cause any visible effect. The optimal concentration of the homologous antisera was in all cases about 30 per cent. The exposure time to serum varied from 15 minutes to several hours. Prolonged treatment in strong antiserum solution often led to a shrinking and bursting of the jelly layer; consequently the eggs were often left completely denuded. If the serum treatment was interrupted before the jelly had been broken up, the precipitate on the jelly surface disappeared and it became invisible, as it is normally.

Fig. 1. Unfertilized eggs of *P. lividus* in sea water containing sucrose and homologous antiserum (concn. 30 per cent). Photograph taken after 15 minutes treatment. For explanation see text.
Fig. 2. Unfertilized eggs of *P. lividus* in sea water containing sucrose and homologous antiserum (concn. 30 per cent). Photograph taken after 40 minutes treatment. For explanation see text.
Fig. 3. Unfertilized eggs of *P. lividus* in sea water after 40 minutes treatment in a sea water sucrose mixture with homologous antiserum (concn. 30 per cent). The eggs transferred into sea water. Photograph taken after 60 minutes.
Fig. 4. Unfertilized eggs of *A. lixula* in sea water containing homologous antiserum (25 per cent) but no sucrose. The jelly precipitated and shrunken, membrane elevated from the cell surface.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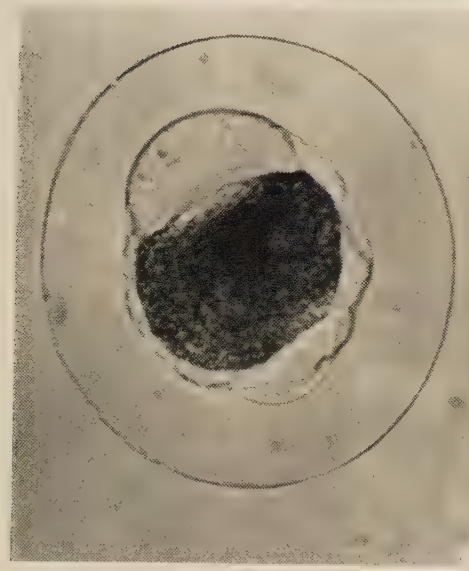
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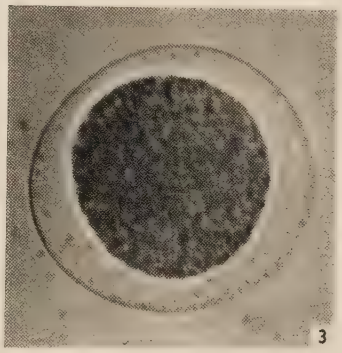
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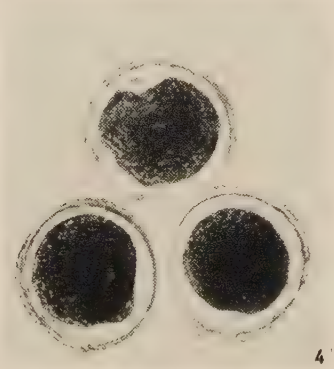
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To obtain a coagulation of the jelly layer, only mature, fertilizable eggs could be used. With underripe eggs no reproducible results were obtained. If the eggs were overripe, or had cytolized, a precipitation of the jelly was obtained with all kinds of sera, even control sera.

Besides the effect on the jelly, the homologous antisera also altered the cell surface, and had a pronounced influence on the entire egg cell. These changes occurred without the addition of sucrose, although the lowering of the electrolyte concentration seemed to promote the antiserum action even in these cases. When the eggs were treated with their homologous antisera in the concentrations mentioned above, a wrinkling of the egg surface could be observed after 30 minutes. The eggs shrank very strongly; gradually a pigment-free zone formed at the surface. Still later a membrane was elevated from the surface. Sometimes the membrane remained attached to the surface at a few points (Fig. 2), but often also a regular, smooth membrane was formed around the egg (Figs. 3, 4). The cortical granules remained intact inside the membrane. Apparently a parthenogenetic activation of the egg had taken place. The membrane formation was characteristically accompanied by changes in the optical properties of the protoplasma, migration of the nucleus to the center, and the subsequent disappearance of the nuclear membrane. However, division of the nucleus and of the cell was seen only rarely and was always abnormal.

The species specificity of the surface-influencing antisera was similar to that involved in the jelly precipitating effect (Table I). The activating action took place even on eggs, which previously had been freed from their jelly layers by treatment with acid sea water at pH 5. The effect on the surface of the eggs was as a rule more independent of the maturity of the eggs than the jelly-precipitating effect. Control sera never induced any visible changes on the egg surface. Eggs treated with homologous antisera at optimal concentration (about 30 per cent) for 30 minutes or more, could no longer be fertilized even after thorough washing.

Preliminary experiments with antisperm- and anti-jelly-sera, and antisera against the soluble part of the egg homogenate (*see Material and methods*) from *P. lividus*, showed that all of them attacked the cell surface in the manner described above (wrinkling, pigment free zone, shrinkage). Activation (membrane elevation etc.) seemed, however, to occur only with antisera against suspensions of the total egg material. No activation could be obtained with the other sera even when used in rather high concentrations. However, this matter must be subjected to further investigations before anything can be stated with certainty.

DISCUSSION

The jelly layer of the sea urchin egg consists of a polysaccharide, combined with sulfuric acid, and containing nitrogenous material. As previously reported (2) the jelly solution contains at least one antigenic fraction identical with a soluble antigenic fraction from the egg homogenate and from the sperm. This antigen seems to be a protein. It cannot yet be stated whether the antigen(s), responsible for the jelly coagulation induced by antibodies, is (are) identical with that (those) found in the *in vitro* experiments. The results reported above seem to point to the possibility that the antigen involved is a substance secreted from the egg in varying amounts, dependent on the state of maturity of the cell. The polysaccharidic component of the jelly might, however, be involved, as e.g. in the case of the *Pneumococcus* capsules, which coagulate in specific antisera. The fact that the coagulation takes place (or becomes visible) only if the electrolyte concentration of the medium is lowered, is consistent with earlier experimental evidence (6). The lack of precipitate in pure sea water is in this special case probably caused by a certain dissociation at increased ionic strength of the antigen-antibody complex.

The specific antibody action on the cell surface opens a possibility for a further study of the nature of its components. Of greatest interest is the parthenogenetic activation caused by the antisera against whole-egg suspensions. If one can assume that no diffusion of the antibody molecules into the interior of the egg is taking place, then the "activation-inhibiting" substance(s) is (are) situated within or very near the surface. By reacting with its (their) specific antibody(ies), a chain of enzymic reactions might be set to work, and thus an activation would start. As an analogy the activation of enzyme systems caused by specific reactions in anaphylaxis and allergy should be mentioned here; see also (4).

SUMMARY

Eggs of the sea urchins *Paracentrotus lividus, Psammechinus microtuberculatus,* and *Arbacia lixula* were treated with homologous rabbit antisera. If the electrolyte content of the medium was lower than that of ordinary sea water, a coagulation of the jelly could be observed. Furthermore, the cell surface was attacked specifically. Antisera for suspensions of whole cells caused a parthenogenetic activation of the eggs. Control sera from untreated rabbits had no visible effect. The reactions observed were species-specific.

This work was supported by a grant from the Swedish Science Research Council and from the Liljevalch Foundation at the Science Faculty, Stockholm University. I am greatly indebted to Professor John Runnström for his encouragement and valuable advice and to the staff of the Stazione Zoologica for their help during my stay at Naples.

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RE-FERTILIZATION OF THE SEA URCHIN EGG

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Normally the fertilized sea urchin egg cannot be re-fertilized. Under experimental conditions Sugiyama (14) has shown that it is possible to refertilize sea urchin eggs even at the two-cell stage. However, in his experiments the treatment rendering the eggs susceptible to re-fertilization has been performed in immediate connection with the first insemination. The present authors have worked out methods whereby this treatment can be performed at any time in the early development. We have also been able to correlate visible alterations of the egg surface with the response to insemination.

This investigation was carried out at the Zoological Station in Naples during February–May 1953. Some experiments have also been made with sea urchins from the Swedish west coast. The following sea urchins were used: *Paracentrotus lividus*, *Psammechinus microtuberculatus*, *Arbacia lixula*, and from the Swedish west coast, *Echinocardium cordatum* and *Psammechinus miliaris*. Most experiments were performed with *Paracentrotus lividus*, in which observations on the chromosomes were most readily carried out. No species specific variability was noted.

EXPERIMENTAL

It is a well-known fact that the vitelline membrane, one of the precursors of the fertilization membrane (9), is very sensitive to the action of trypsin (11). As it was necessary to eliminate the fertilization membrane the eggs were pretreated in a 0.0005 per cent solution of crystalline trypsin. The time of exposure to trypsin must be as short as possible (as a rule 5–10 minutes) to avoid damaging the egg surface. Too long an exposure results in polyspermic fertilization. After pretreatment with trypsin the eggs were washed in normal sea water. Fertilization was performed at a sperm-concentration of 2.6×10^6/ml.

The fertilized denuded eggs were treated with Ca-free, Ca-Mg-free and Mg-free media at different intervals after the first insemination. The time of treatment was 5 or 10 minutes; the time must not be longer than 15 minutes, otherwise the eggs are injured. After this treatment the eggs were transferred into sea water and re-inseminated (2.6×10^6 spermatozoa per ml). The artificial sea water was prepared according to Harvey (3) and Ca^{+2} and Mg^{+2} were replaced by Na^+. The re-fertilized cultures were examined for polyspermy between the first and the third cell division. About 200–250 eggs were counted in every culture. The occurrence of re-fertilization was detected using a Leitz' phase contrast microscope or by staining the larvae with aceto-carmine.

Twenty-five experiments were carried out by this method. The results show that it is possible to obtain re-fertilization even when the treatment with media lacking divalent cations is not performed in immediate relation to the first insemination. Some of these results are presented in Table I.

Sugiyama (14) states: "Ca-Mg-free sea water becomes ineffective when any of Ca^{++}, Mg^{++}, Sr^{++}, or Ba^{++} is added. It may be concluded that those media which contain any divalent cation are ineffective." In all our experiments we have exposed different lots of the egg-samples to Ca-Mg-free, Ca-free and Mg-free solutions. We have not, however, been able to establish any definite difference between these media in their action on the egg-surface. They all facilitate re-fertilization but Mg-free medium has perhaps a weaker effect than the others.

After treatment with media lacking divalent cations the hyaline layer was very swollen. If the treatment lasted too long the blastomeres were entirely separated although the cells continued to cleave and formed cellplates (5).

Table I

Results of fertilization of *Paracentrotus lividus* eggs pretreated with crystalline trypsin (Armour Co.).
Untreated eggs 95–100 per cent fertilization.

Control. Inseminated; then re-inseminated. Polyspermic eggs per cent	Eggs inseminated; then treated with:			Time from the first to the second insemination (minutes)
	Ca-Mg-free sea water; then re-inseminated. Polyspermic eggs per cent	Ca-free sea water; then re-inseminated. Polyspermic eggs per cent	Mg-free sea water; then re-inseminated. Polyspermic eggs per cent	
6	83	91	91	10
5	70	91	94	20
1	58	53	69	30
8	35	34	16	40
3	12	5	6	50
5	3	1	4	75

In cultures where treatment and re-insemination were carried out at about the first cell division there was a decrease in the number of re-fertilized eggs (cf. Table I, the two last exps.). It seems to be more difficult to obtain re-fertilization during the nuclear division.

We have also tried to remove the vitelline membrane with crystalline chymotrypsin. A concentration of 0.0006–0.0009 per cent applied for 10 minutes easily inhibited the formation of a fertilization membrane without injuring the egg. Three to five minutes after the first insemination sodium-lauryl-sulfonate was added to give a final concentration of 0.0005 per cent. This treatment momentarily inactivates the spermatozoa. No abnormality in the development follows this treatment. The hyaline layer does not dissolve and cleavage is quite normal. For the action of detergents on the egg cf. (13). If higher concentrations of detergent are used, there is a remarkable swelling of the hyaline layer. However, it is not possible to re-fertilize the eggs without subsequent treatment with Ca-Mg-free, etc., media.

After treating with chymotrypsin and detergent the eggs were subjected to Ca-Mg-free, etc., media and re-inseminated. About 30 experiments were performed according to this method and the results obtained are very uniform (Table II).

In these experiments a sperm concentration of 1.3×10^6/ml was employed. The larvae, which had not become polyspermic, showed normal development.

TABLE II

Results of fertilization of *Paracentrotus lividus* eggs pretreated with crystalline chymotrypsin (Worthington Biochem. Lab.).
The spermatozoa inactivated 3 minutes after the first insemination.
Untreated eggs 100 per cent fertilization.

Control I. Inseminated. Polyspermic eggs per cent	Control II. Inseminated; then re-inseminated. Polyspermic eggs per cent	Eggs inseminated; then treated with Ca-free sea water 10 minutes before the second insemination. Polyspermic eggs per cent	Time from the first to the second insemination (minutes)
1	1	31	35
2	2	40	45
2	8	57	45
2	3	12	55
2	2	10	65

Inhibition of membrane formation with chymotrypsin seems to be the preferable method for it leaves the eggs intact. Mere re-insemination of the denuded eggs gives almost no polyspermy, whereas if the eggs have been exposed to Ca-free sea water there is heavy polyspermy. Also in these experiments it was difficult to obtain re-fertilization during the nuclear division (Table II, the two last exps.).

In some experiments we have been able to carry out re-fertilization even after the second cell division. As a rule only one of the blastomeres became polyspermic and the percentage of irregular larvae was about 15–25.

Some experiments with lysozyme were also carried out. The eggs were fertilized in the presence of 0.01–0.05 per cent crystalline lysozyme. Membrane-formation was delayed and only a few granular membranes occurred (cf. 12). Rather high percentages of polyspermic eggs (ordinarily 10–30 per cent but in one exp. 59 per cent) were obtained. Re-insemination of the eggs 30–40 minutes after the first insemination gave no significant differences in the percentage of polyspermic eggs. No treatment with Ca-free medium, trypsin or chymotrypsin was applied in connection with lysozyme.

DISCUSSION

The attachment of the spermatozoon to the egg surface induces a wave, which begins at the point of sperm-entry and sweeps over the egg. As this wave progresses, it renders the egg immune to the entry of additional spermatozoa (4). The old opinion that the fertilization-membrane blocks polyspermy is no longer tenable for it has been repeatedly shown, that membrane-freed eggs cannot be re-fertilized (14).

Runnström showed that the cortical wave is accompanied by a colour-change, easily detectable with dark ground illumination (8). The time of conduction of this impulse is about 20–30 seconds in *Psammechinus miliaris* and this time is regarded as sufficiently short to protect against polyspermy (7). *Psammechinus miliaris* has a rather low rate of fertilization and this time may be shorter in other species (2). The entrance of the spermatozoa is followed by the formation of the reception cone and the hyaline layer. The latter becomes visible after 20–30 seconds (6), and the present authors have also confirmed this.

In the oocyte there is a hyaline layer present which upon fertilization forms hyaline protrusions (10). The fertilization is polyspermic for the oocyte and cannot develop any block to the supernumerary spermatozoa. The hyaline protrusions which form may be equivalent to the reception cone of the mature egg. No cortical granules are found in the cortex of the oocyte, but Runnström and Monné (10) have observed that the granules are present in the interior of the cytoplasm, and migrate toward the surface during the maturation process.

The hyaline layer of the ripe egg is perhaps masked by the cortical granules. As soon as the granules are expelled a thin hyaline layer becomes visible and in the course of 10–15 minutes it attains a thickness of about 2 microns.

In all our experiments we have observed visible injury to the hyaline layer after treatment with Ca-Mg-free, etc., media. If the hyaline layer was entirely dissolved heavy polyspermy occurred upon insemination; if there was only a slight swelling of the layer only a low percentage of polyspermy was obtained. The hyaline layer is not necessary for cell division, although blastulae are not formed in the absence of this layer (5). Therefore, it seems reasonable to consider the hyaline layer (or part of it) as constituting the block to polyspermy. The cortical granules, however, seem also to be necessary to establish this block. Runnström's work on the behaviour of the cortical material (9) has recently been confirmed and completed by Endo (1). The latter has shown that the Ca-ion is indispensible for the formation of the "rods", as well as for the hardening of the fertilization membrane. He also suggests, "that the substance responsible for the hardening of the membrane is the same as the substance of the rods". Since neither the membrane nor the rods dissolve in Ca-free medium, there appears to be a definite difference between the rods (and the membrane) on one side and the hyaline layer on the other.

The cortical granules are precipitated by lysozyme (12). Since lysozyme is a basic protein this precipitation may be due to a reaction between this protein and acid components present in the cortical granules. Clupein, another basic protein, has also been demonstrated to precipitate the cortical granules and in addition the eggs become polyspermic upon insemination (15). It seems reasonable to suggest that the precipitation of the cortical granules prevents an action which is normally exerted on the egg surface by the content of these granules. The precipitation of the granules would thus establish conditions in the mature egg resembling those prevailing in the oocyte. In both cases no block to polyspermy would be formed. On the other hand lysozyme may, besides its action on the cortical granules, interfere with the properties of the egg surface so as to inhibit the formation of the block to polyspermy.

SUMMARY

A method is described by means of which fertilized sea urchin eggs can be re-fertilized at various intervals after the first insemination. During the nuclear division the possibility for additional sperm to enter the egg is diminished.

Further experiments have indicated causal relationship of the breakdown of the cortical granules and the formation of the hyaline layer to the establishment of the block to polyspermy.

The authors wish to express their great indebtedness to Professor John Runnström for interest and valuable discussions. Thanks are also due to Dr. R. Dohrn, Naples, and Dr. G. Gustafson, Kristineberg, for supply of material. This work was supported by the Swedish Natural Science Research Council.

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THE STRUCTURE OF CHLOROPLASTS

III. A STUDY OF PYRENOIDS

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In 1883 Schmitz published the results of a comprehensive microscopical investigation of the chromatophores of algae (12), and in the same report pyrenoids are also treated in considerable detail. The author describes them as spherical bodies enclosed in the chromatophore similar to the nucleolus in the cell nucleus. These pyrenoids consist of a very dense and strongly refractive substance, mostly surrounded by one or several layers of tightly packed starch granules. According to Schmitz, pyrenoids are to be found in all *Chlorophyta* with the exception of a few named genera but they are probably lacking in all *Phaeophyta,* whilst they may appear in a few genera in *Rhodophyta;* also each of the green cells of the primitive moss *Anthoceros* has a pyrenoid, which has been described by McAllister (9, 10) and other investigators. McAllister also gives a short review of other published informations on pyrenoids. Lutman (8) and Carter (1, 2) have investigated the chromatophores of many species of *Closterium* which will be further described in this report. Steinmann (14) has published electron micrographs of the chloroplast and pyrenoid of *Spirogyra*, and Wolken and Palade (15, 16) have made an electron microscopical study of the chromatophores of *Euglena* and *Poteriochromonas.* Their micrographs of *Euglena* show the pyrenoid as a dense body lamellate rather similar to the remainder of the chromatophore.

All published observations indicate that the pyrenoids are centres of a concentrated production of starch and it may be assumed that they contain, in a concentration higher than the rest of the chromatophore, some or all of those enzymes which perform the assimilation of carbon dioxide. Because the pyrenoids are much denser than the enveloping chromatophore, the possibility of their bulk-isolation from convenient sources may be of considerable significance, especially for the purpose of studying the assimilation of carbon dioxide.

Pyrenoids are found only in plants whose chloroplasts do not contain the well known grana. The question readily arises whether or not there is any connection as to function or phylogenesis between the grana of archegoniates and phanerogams on one side and the pyrenoids of algae on the other. These aspects led to the investigation forming the subject of the present report.

So far, various of the structure of pyrenoids with varying physiological conditions have not been investigated systematically. The subject of the present work is confined to the general features of the structure of pyrenoids.

MATERIAL

Sections of the green algae *Enteromorpha intestinalis, Cladophora glomerata, Spirogyra sp., Mougeotia sp.,* and *Closterium acerosum* and *lunula* were investigated by means of electron microscopy.[1] In particular chloroplasts were studied and special attention was devoted to the structure of the pyrenoids. *Closterium* was chosen because of the large size of its pyrenoids. With the exception of *Cladophora* all the algae studied were obtained from natural sources and not from pure cultures. The specimen of *Cladophora* was from the Collection of Algae and Protozoa, Cambridge, England.[1] During the winter bottled material was used which had been collected during the summer.

METHODS

For fixation osmium tetroxide was used in buffered and hypertonic solutions (11, 15) as well as in the form of a vapor. The vaporisation method gives the best results probably because the concentration of fixative in this case increases slowly and continuously. After dehydration with alcohol the specimens were embedded in a mixture of 3 parts methyl- and 7 parts butylmethacrylate; the polymerisation was catalyzed by 2 per cent 2,4-dichlorobenzoylperoxide.

Sectioning was accomplished with a standard Spencer microtome, modified by the removal of the device for mechanical feeding which was replaced by an electrothermal feeding unit. This (*a* in Fig. 1) was made in principle according to Eden, Pratt, and Kahler (3) but was machined so that it could replace the original horizontal feeding mechanism containing the specimen holder. There are some disadvantages in using thermal expansion in combination with a reciprocating movement for sectioning. One of these is that the specimen contacts the edge of the knife also in the return non-cutting stroke. To reduce the effect of this imperfection the specimen was arranged to meet the knife edge near the lowest point of its vertical path. This was achieved by means of a special type of brass block for insertion of the specimen (Fig. 2).

The microtome was motor-driven and made one cutting cycle in 2 seconds. The sections were floated directly from the knife edge on to a liquid surface (4), usually a mixture of 25 per cent ethyl alcohol in water. Schick razor blades were used as knives and were sharpened against a glass surface with Linde B polishing powder suspended in soap solution (5, 13).[2]

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[1] I am indebted to Professor H. Skuja, Uppsala, for furnishing me with some of the material and for determining those species, and to Dr M. Waern from whom *Cladophora* was obtained.

[2] I wish to acknowledge the helpful information about these techniques from Dr F. Sjöstrand, Stockholm.

In separate experiments the sharpening of the razor blades was studied to find optimum conditions for the smoothest possible edge. The pressure on the blade during sharpening was maintained constant and the total length of blade traverse on the glass surface during the grinding process was varied. By subsequent observations in the electron microscope of fragments of the sharpened razor blades the optimum value of total traverse was determined and was found to vary but little from blade to blade.

The sections were studied at a magnification of 8500 in a modified Siemens electron microscope which was run at 90 kV. The embedding medium was not removed (6).

RESULTS

The main results of the investigation are given in the Figs. 3–13.

The extra-pyrenoid substance of the chloroplast. The chloroplasts of the algae appeared to consist of several very thin undulating membranes separated by rather large interspaces and extended in the direction of the major axis of the chloroplasts (Fig. 3). The thickness of the membranes was measured in the electron micrographs as 80 ± 20 Å. Also more remote from the pyrenoid starch grains of different size were frequently to be found in the chloroplast. The long chloroplast of *Cladophora* and *Closterium* appeared to be constricted at approximately equidistant intervals. Chromatophores usually multiply through fission; however, Schmitz (12) has reported that such dividing chromatophores in some cases do not completely separate but present a beaded appearance. This was observed by Schmitz in *Cladophora*, *Vallonia*, and *Chaetomorpha*. Such chains of small chromatophores can be seen also in phanerogams under certain conditions (Leyon: unpublished observations on *Taraxacum* and *Solanum tuberosum*).

The pyrenoid. Enteromorpha and *Cladophora* were fixed and embedded during the winter months February and March and during storage this material was exposed to subdued daylight from a window. The pyrenoids in these two preparations appeared as oval very dense bodies about 2 μ long and 1 μ broad, in which no laminar structure was clearly definable. There was a surrounding layer of starch about as thick as the pyrenoid itself and enclosing it in the form of two hollow hemispheres. The extremities of the pyrenoid were connected to the chloroplast outside the starch layer.

In *Spirogyra* which was derived from a natural source in July, the laminar structure of the pyrenoid was clearly seen (Fig. 6). The laminae of the pyrenoid appeared to emerge through the starch layer and extend into the enveloping substance of the chloroplast where they were subdivided into the membraneous structure previously described.

Closterium acerosum which had been kept in weak light for a few months contained pyrenoids surrounded by several layers of starch granules (Fig. 5). The interior of the pyrenoid showed a dense structure consisting of tightly packed lamellae. When exposed to daylight the algae migrated into small clusters toward the lightest parts of the vessel. Such closteria contained pyrenoids without starch (Fig. 9). Fig. 7 shows part of the pyrenoid of *Closterium lunula* which was fixed and embedded in July and can be seen to be surrounded by large starch granules and with lamellae arranged in a rather complicated way. A model of such a pyrenoid could be made by reassembling the pieces cut from an onion with their section-surfaces turned outwards. — The thickness of the pyrenoid laminae were measured from Fig. 9 as varying from 300–700 Å. There may also be a variation in thickness with different physiological conditions.

In many sections of *Closterium* the connections between the pyrenoid and its enveloping substance could be studied. Usually 3, 4, or even 5 fine laminae of the chloroplast emerged into a single thick lamella of the pyrenoid. The possibility could not be excluded that the thin laminae of the chloroplast retain their identity within the pyrenoid (Fig. 8). If so, some other substance must be included in the lamellae of the pyrenoid in order to account for its large density. — There is some question as to what degree the osmium fixative contributes to this density.

Chlorophyll in pyrenoids. Since the pyrenoids probably form part of the carbon dioxide assimilating mechanism of the plant, it is of great interest whether or not they contain chlorophyll. Schmitz (12) is convinced from his own observations that this is not the case and is of the opinion that some earlier authors who described the pyrenoids as chlorophyll-containing bodies were misled by the high density of the pyrenoids which make them appear as dark bodies within the chloroplasts. It must be assumed that all these observations were made on whole cells since neither Schmitz nor other authors mention any sectioning or dissection. In that case, however, the decision as to the colour of the inner structure of the pyrenoid may lead to some ambiguity.

By simple microdissection under a binocular at a magnification of 100 × the colour of the large pyrenoids of *Closterium acerosum* was investigated. With some care it was possible to disrupt the cell wall with thin glass rods and extrude part of the contents of the cell. However, it was not possible to

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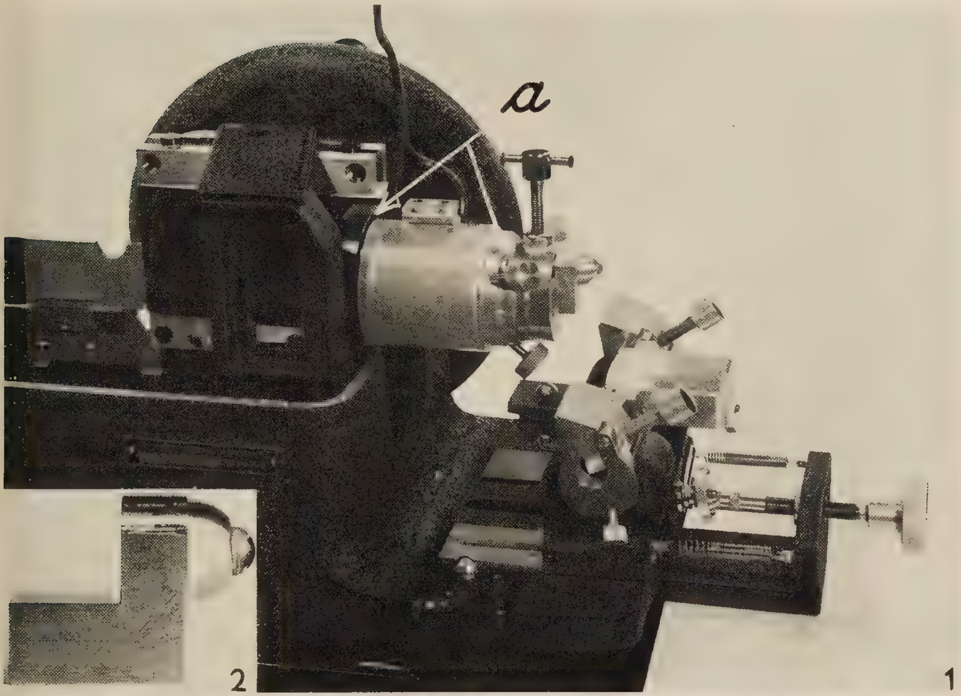
Fig. 1. The modified Spencer microtome with thermal expansion device (a).
Fig. 2. Brass block for specimen insertion.
Fig. 3. Section through part of the cell of *Closterium acerosum*. From right to left: cell wall cytoplasm, part of the laminar chloroplast, cytoplasm.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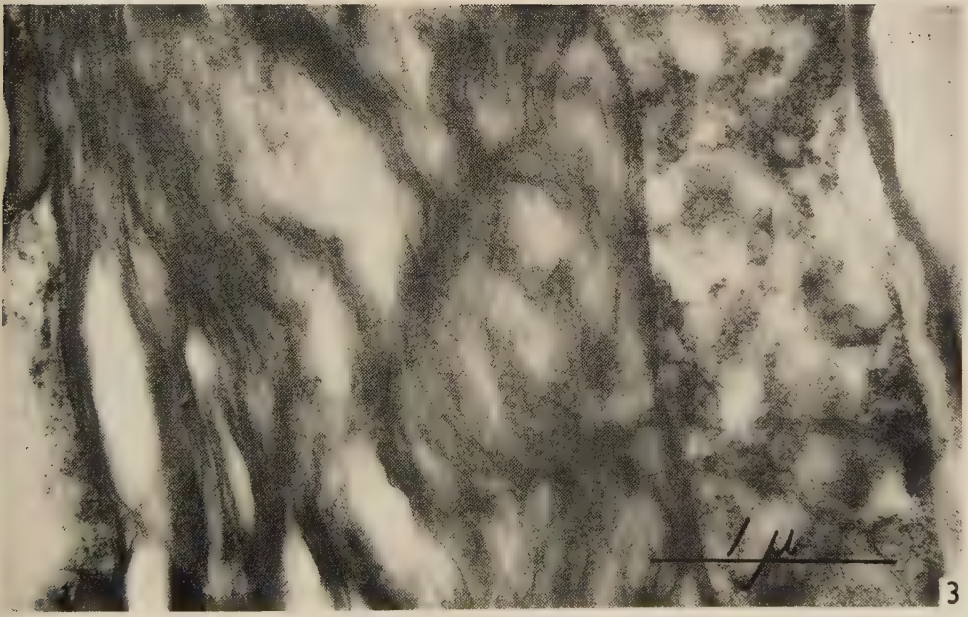
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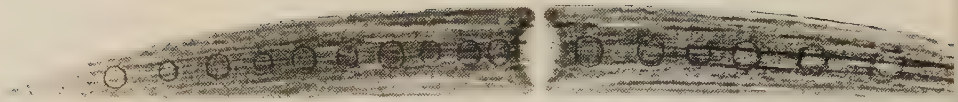

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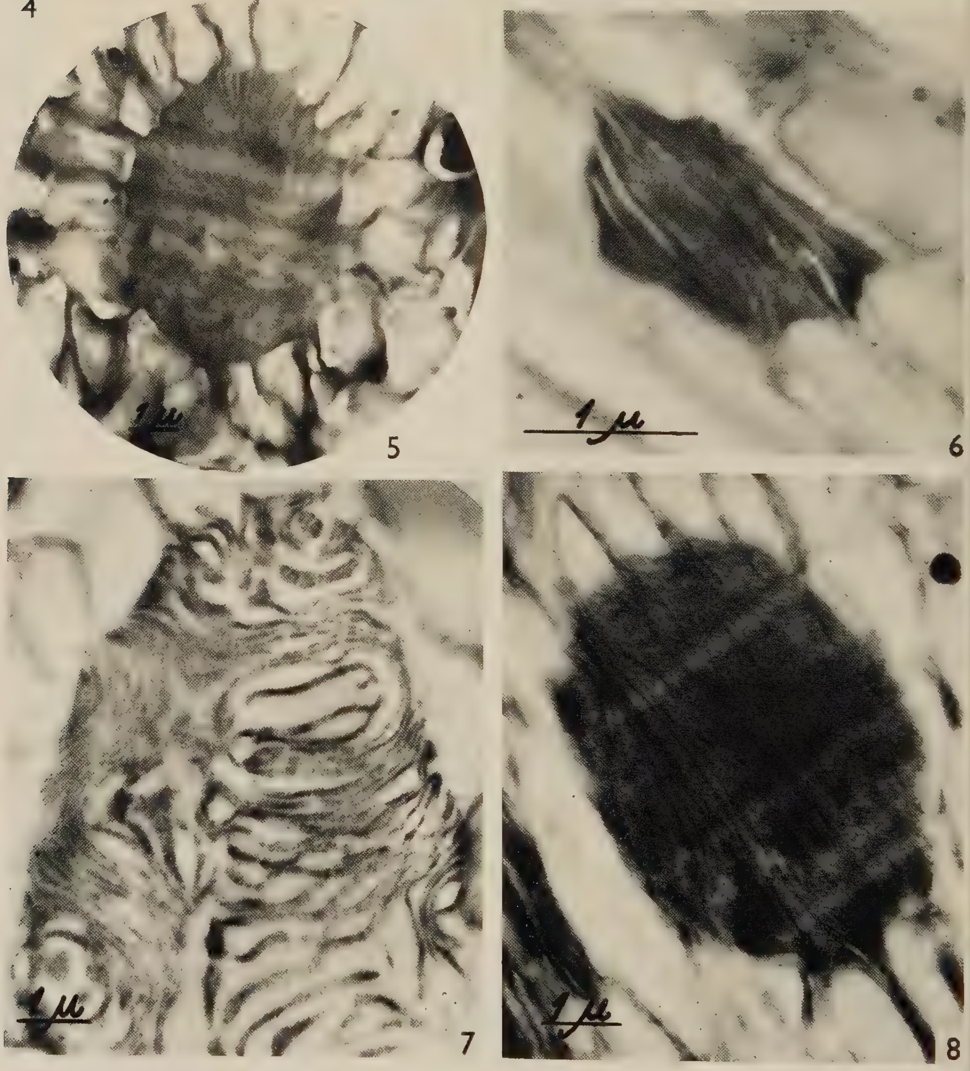
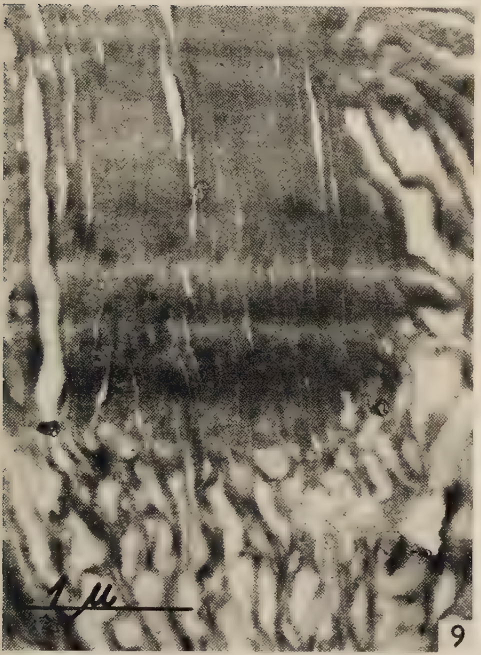


Fig. 4. Pyrenoids in *Closterium acerosum* (570 ×).

Fig. 5–8. Sections of pyrenoids surrounded by starch grains. Figures 5 and 8 are from *Closterium acerosum*, 6 from *Spirogyra* and 7 from *Closterium lunula*. Fig. 7 shows the arrangement of the laminae in part of the pyrenoid. In Fig. 8 the laminae of the chloroplast can be seen traversing the pyrenoid.



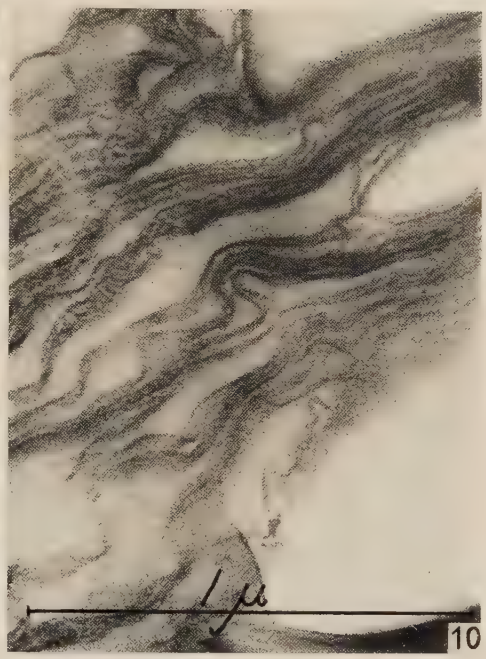


Fig. 9. Section of part of the pyrenoid (*Closterium acerosum*) without starch.
Fig. 10. Near the margin of the pyrenoid its lamellae (left) split into thinner membranes which extend between the starch grains into the extra-pyrenoid laminar system of the chloroplast.

segregate the pyrenoids from the viscous green mass thus obtained. After the algae had been fixed in formaldehyde (2 per cent, $\frac{1}{2}$ hr), the dissection was repeated and some pyrenoid bodies were extracted from the rest of the cell contents. Under the microscope, their colour was red — probably due to fluorescence. By slight pressure on the cover glass they were transformed into a thin smear which showed a green colour.[1]

DISCUSSION

Some earlier authors have claimed that the pyrenoid substance itself is transformed into starch. However, from later information it must be concluded that the pyrenoids are more or less permanent cell organellae which produce starch intensely but still retain their identity. The consideration of the pyrenoids as organellae is consistent with their ability to multiply through fission.

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[1] Note added in proof: This result has been confirmed by Dr Karin Aschan, Uppsala who isolated pyrenoids from *Closterium acerosum* by micromanipulation.

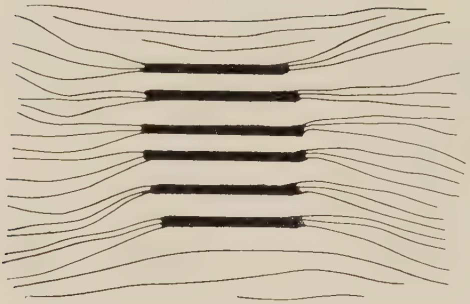

Fig. 11.

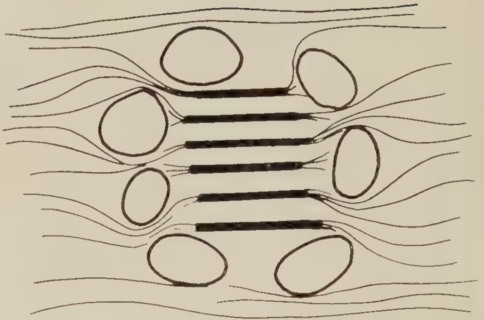

Fig. 12.

Fig. 11–13. Schematic diagrams of pyrenoids.

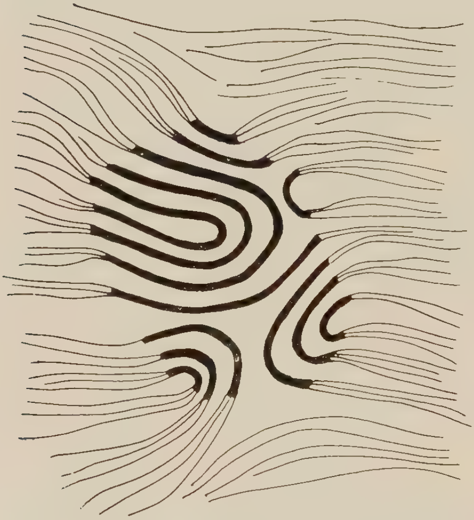

Fig. 13.

11. Simple pyrenoid without starch (*Spirogyra*, *Mougeotia*).
12. Simple pyrenoid with starch.
13. Pyrenoid of *Closterium* without starch.

An investigation of grana in the phanerogam chloroplast initiated this study of pyrenoids. Previously Schmitz (12) stated that pyrenoids are present only in *Thallophyta* and the primitive moss *Anthoceros* but lacking in all other archegoniates and in phanerogams. He then inquired significantly as to whether the chromatophores had lost their pyrenoids during evolution or if the pyrenoids were possibly present also in archegoniates and phanerogams but were too finely distributed to be discerned at that time.

The well-known pyrenoid of *Anthoceros* (9, 10) seems to be of particular interest in this respect. It is reported to consist of several small units (25 — several hundreds according to McAllister) which may be an aggregate central mass within the chloroplast or may be distributed throughout the whole chloroplast as small separate producers of starch. Which state prevails depends on the physiological conditions and the age of the cell. In *Notothylas* a similar pyrenoid is reported to be found.

The question arises as to the possibility of this type of pyrenoid being intermediary between the typical pyrenoid of algae and the grana of the phanerogams.

SUMMARY

1. Electron micrographs of the pyrenoids of *Spirogyra* and the chloroplast and pyrenoids of *Closterium* are presented. The chloroplasts and their pyrenoids of *Enteromorpha*, *Mougeotia*, and *Closterium* have also been investigated.

2. The chloroplasts of the investigated algae were found to consist of a membraneous structure with laminae whose thickness were measured from the micrographs as 80 ± 20 Å.

3. The pyrenoids have a lamellar structure. Their lamellae are thicker than those of the remainder of the chloroplast. Each lamella of the pyrenoid is connected with, or may be traversed by, some of the laminae of the chloroplast. Starch appears between these connections.

4. The pyrenoid of *Closterium acerosum* was investigated with respect to chlorophyll content and was found to be green.

5. The question is raised as to the correspondence of the pyrenoids to the grana of the phanerogam plastids.

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METHYL-MERCURY-CHLORIDE AS A SPECIFIC REAGENT FOR PROTEIN-BOUND SULFHYDRYL GROUPS

ELECTRON STAINS II

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Fixation with osmium tetroxide at a suitable pH gives very good preservation of the tissue structures as well as high contrast images in electron microscopy (23). However, little is known of the exact nature of the chemical reaction between osmium tetroxide and the many organic components in tissues (3, 9, 26). The localization of certain chemical compounds in electron microscopical preparations is possible by binding heavy metals to these compounds, thus increasing their electron scattering. Such "electron stains" for specific groups in the protein side chain have been suggested by Lamb *et al.* (21), and gallocyanine-indium-alum has been proposed as an "electron stain" for nucleic acids (2).

Sulfhydryl groups are known to play fundamental roles during the process of keratinization (14), and in enzyme activity (22). Several histochemical methods with considerable specificity for the demonstration of protein-bound SH-groups in tissues have been reported (4, 6, 7, 8, 10, 24). The coloured end products of these reactions can be seen with the aid of the light microscope, but these methods are inapplicable for electron microscopy. However, no specific reagents have been reported for the localization of SH-groups in the tissues by electron microscopy.

Mercury compounds with the principal configuration $R - Hg - X$ (where R represents an organic radical which is bound to a cation, X, by the Hg-atom) show high selective reactivity with protein-bound SH-groups. Schematically this reaction follows the equation:

$$R - Hg - X + HS - R = R - Hg - S - R + XH.$$

Mercury compounds of this type have been widely used in biochemistry for the quantitative determination of SH-groups. Hellerman *et al.* (18) used phenyl-mercury-hydroxide for a complete blocking of the enzymatically active SH-groups in urease. The blockade could be reversed by H_2S. Benzyl-mercury-chloride, phenyl-mercury-chloride and chlor-methyl-mercury-chloride also showed the same reactivity with available SH-groups. These compounds had no oxidizing effect. P-chloro-mercury-benzoate was used by Anson (1) for the blocking and quantitative determination of the SH-groups in egg-albumin. The same compound was employed by Hellerman *et al.* (17) for further studies of the SH-groups in urease, by Friedenwald and Herrmann (13) for studies of the SH-groups in amin-oxydase and by Singer and Barron (27) for investigation of a number of different enzymes. The latter authors showed that the SH-activity could be reversed by glutathione. The equilibrium constant for the reaction of serum albumin with methyl-mercury-iodide as well as of other proteins with methyl-mercury-nitrate was determined by Hughes (20). These investigations have shown that *simple mercury compounds* (of the configuration R — Hg — X) *react quantitatively with protein-bound, available SH-groups,* e.g., in enzymes and in denatured egg albumin.

Mercury reagents have also been employed for the histochemical localization of protein-bound SH-groups in biological material. Bennett (6) utilized a combination of mercury with an azo residue [1—(4-chloro-mercury-phenyl-azo)-napthol-2] to reveal the sites for the mercury-sulfhydryl reactions in light microscopy. He also emphasized that there may be a possibility of unspecific reactions due to the complex structure of the azo-component. The great similarity of this reagent and certain lipid stains, such as Sudan black was pointed out by Pearse (25).

A mercury reagent with a *methyl* radical will give the least possibility for side reactions and therefore methyl-mercury-chloride was regarded the most suitable reagent ("electron stain") for SH-groups in tissues. This paper describes the biochemical properties of this compound in relation to the SH-groups in some biological material. A preliminary introduction to the application of this reagent for electron microscopical purposes will also be given.

EXPERIMENTAL

The methyl-mercury-chloride (MMC) was synthesized according to Hilpert and Ditmar (19). Twenty-five gm $HgCl_2$ was dissolved in 130 ml 10 per cent HCl. The solution was heated to 90°C, and 25 gm technical Al_4C_3 was then added in small portions with vigorous stirring. Pure MMC was obtained by steam distillation as small fatty platelets and grey particles in the distilled aqueous fluid. The distillate was filtered and the residue was dissolved in absolute alcohol. The filtrate, calculated from the figures given by Slotta and Jacobi (28), contained 0.2 mg MMC in 100 ml of the solution, i.e., 0.00001 molar. This solution diluted 1:2500 gave a positive dithizon reaction for mercury [5 ml of the sample plus 0.5 ml reagent (2 mg diphenyl-thio-carbazone in 100 ml carbon-tetra-chloride)]. This reaction is sensitive to quantities of Hg as low as 0.24_γ (12). An alcoholic solution gave a positive dithizon reaction at a dilution of 1:1500.

The MMC was also prepared by the Grignard method (28). Twelve gm magnesium turnings in 300 ml cooled absolute ether were treated with 60 gm cooled methyl-bromide in small portions. The resulting Grignard solution was heated on a water-bath under a reflux-cooler for half an hour, and then carefully filtered through glass-wool. While vigorously shaking the filtrate, 200 gm powdered mercury-bromide was added, and the mixture boiled for another half hour. When cooled, 20 ml water was added and the ether was distilled off. Another 100 ml of water was then added, and the product was sucked dry on a Büchner filter. The product, methyl-mercury-bromide, was then boiled with 1 per cent hydrochloric acid, filtered, and the process repeated; Yield: 127 gm equal to 89 per cent of the theoretical. To 36 gm of the methyl-mercury-bromide in 400 ml ethanol at 40°C, 60 ml of a 40 per cent sodium hydroxide solution was slowly added, without heating (heating resulted in a precipitate, partly consisting of pure mercury). The residue was filtered off, and the solution was neutralized with 2N hydrochloric acid, using phenolphtaleine as an indicator. The neutralized solution was poured into 4 liters of ice water and sucked dry on a Büchner filter. Yield: 21 gm, white, crystalline leaflets of MMC. The chloride compound has a higher solubility in water as well as in alcohol than corresponding bromide or iodine compounds. The presence of a methyl group results in a more soluble product as compared to higher paraffins.

In order to establish the biochemical properties of the MMC in relation to SH-groups in biological material, the following experiments were performed:

1. To determine the presence of available SH-groups in living tissue.
2. To prove the reaction of MMC with available SH-groups.
3. To determine the specificity of the reaction.
4. To determine the nature of the bond between the mercury reagent and the SH-groups.

1. The presence of SH-groups was demonstrated by means of the nitroprusside reaction, incorporating the slight modifications suggested by Anson (1). Native egg albumin showed a negative nitroprusside reaction, while denatured egg albumin (Bacteriological albumin, Difco) showed a positive but very faint reaction. Small fresh pieces of skin, striated muscle and pancreas from calf gave a positive nitroprusside reaction, indicating the presence of available SH-groups (Table I).

2. The reaction of MMC with free SH-groups was tested in the following way: 10 ml of the aqueous, 0.00001 molar MMC solution was added to 0.1

ml of a 0.001 molar cystein solution. The positive nitroprusside reaction of the latter turned negative upon the addition of MMC. The same result was obtained when 10 ml of the same MMC-solution was added to 1 ml of a 0.0001 molar glutathione solution. Dialysis against water resulted in diffusion of mercury-mercaptides through the membrane, and a positive dithizon reaction in the dialysate.

The reactions of MMC with SH-groups in albumins and in tissues were tested in the following general way: To 20 ml of native egg albumin was added 1.0 or 0.5 ml aqueous, or alcoholic, MMC solution. These mixtures were dialyzed a) immediately, or b) after 12 hours incubation at 37°C, using Visking Corporation dialysis tubes. The mixtures were dialyzed against 100–150 ml distilled water which was frequently stirred with a clean glass rod. Samples from the dialysate were taken after 24 and 48 hours, and tested for the presence of mercury. The nitroprusside reaction was performed on the contents of the tube. The presence of mercury in the dialysate was indicated by a positive dithizon reaction. This indicated that in the native egg albumin, where no SH-groups were available, the added mercury was free to pass through the membrane and could thus be shown in the surrounding fluid. When denatured egg albumin (Difco) was analyzed in the same way, no mercury could be found in the dialysate. At the same time the faint positive nitroprusside reaction of the albumin became negative, thus indicating that the SH-groups had been occupied by the mercury compound. Non-dialyzable mercury-containing molecules had evidently been formed. However, if MMC was added in excess, mercury appeared in the dialysate (Table I).

The same experiments were performed with minced preparations of fresh skin, striated muscle and pancreas from calf. Immediately after their removal from the organism, the tissues were frozen in a carbon-dioxide-acetone mixture at —80°C, and cut into small pieces after the addition of 0.1 molar KCN solution at +4°C. Ten ml of the KCN solution was used for 50 gm of fresh tissue, serving to prevent a catalytic effect of heavy metal salts on the oxidation of sulfhydryl groups by atmospheric oxygen during the cutting procedure. The concentration of the KCN was too low to break up disulfide bonds (1). The tissues were then minced and diluted with equal parts of distilled water. Twenty ml aliquots of these solutions were added to the dialysis tubes, and the aqueous and the alcoholic MMC solutions added as described above. In no case could mercury be found in the dialysate. The results of these experiments are summarized in Table I.

These experiments indicate that the addition of MMC to proteins or pieces

Table I

	Untreated		After treatment with MMC	
	Dithizon reaction in the dialysate	Nitroprusside reaction in the tube	Dithizon	Nitroprusside
Egg albumin, native	–	–	+	–
Egg albumin, Difco, powder	–	(+)	–	–
Skin, calf	–	+ +	–	–
Muscle, calf	–	+ +	–	–
Pancreas, calf	–	+ +	–	–
Cystein, 0.1 ml/0.001 mol.	–	+ + +	+	
Glutathione, 1.0 ml/0.0001 mol.	–	+ + +	+	–

of tissue containing available SH-groups (as indicated by a positive nitroprusside reaction) results in the formation of non-dialyzable mercury compounds. When the mercury reagent was added in excess or in stoechiometric relation to the SH-groups present, the nitroprusside reaction became negative. Addition of MMC to a substance with no available SH-groups resulted in a diffusion of unbound mercury molecules into the dialysate. This was also the case with cysteine and glutathione; diffusable mercury molecules were then formed giving a positive dithizon reaction in the dialysate.

Additional evidence indicating the reactivity of MMC with the SH-groups of albumin and tissue was obtained by employing the denaturating effect of guanidine hydrochloride (15). The denaturating effect of this compound has been widely utilized in the biochemistry of sulfhydryl groups to make all existing SH-groups accessible. Guanidine carbonate (The British Drug Houses, London) was dissolved in a minimum quantity of distilled water. After filtering, equal parts of absolute alcohol were added. The suspension was stored for 12 hours (over night) at +4°C, and was then refiltered at this temperature. The residue was washed in absolute alcohol, and then dissolved in small portions of concentrated hydrochloric acid. The resulting clear solution was slightly acid, and was neutralized by the addition of ammonia (NH_4OH). Two hundred gm of guanidine carbonate yielded 200 ml of a 80 per cent solution of guanidine hydrochloride.

Native egg albumin was diluted in distilled water (1:2), filtered, and dialyzed for 48 hours. The nitroprusside reaction remained negative. To 10 ml of the albumin solution, 5.0 ml of the guanidine hydrochloride solu-

TABLE II

	Guanidine hydrochloride-pretreated samples										
Relative Conc. of MMC	10		5		4		2		1		–
	saturated		aqueous		alcoh.		alcoh.		alcoh.		control
	Hg	SH	Hg	SH	Hg	SH	Hg	SH	Hg	SH	SH
Egg albumin, native	–	–	–	–	–	–	–	+	–	+	+
Egg albumin, Difco, solution	+	–	+	–	+	–	–	–	–	–	+
Skin, calf, minced, 10 ml	+	–	+	–	+	–	+	–	–	–	+
75 ml	–	–	–	–	–	–	–	–	–	–	+
Muscle, calf, minced, 10 ml	+	–	+	–	+	–	–	–	–	–	+
50 ml	–	–	–	–	–	–	–	–	–	–	+
Pancreas, calf, minced, 10 ml	–	–	–	–	–	–	–	–	–	–	+
50 ml	–	–	–	–	–	–	–	–	–	–	+
Guanidine hydrochloride (control)	+		+		+		+		+		–

tion was added. The positive nitroprusside reaction now obtained indicated the presence of available SH-groups. To separate tubes containing the diluted egg albumin, 1.0 ml of either aqueous or alcoholic MMC solution was added, and dialysis was performed for 24 and 48 hours against distilled water. The dithizon reaction was negative in the dialysate, indicating the absence of permeable mercury molecules. The nitroprusside reaction was also negative, indicating that the SH-groups made available by the guanidine hydrochloride were occupied by the mercury reagent.

Addition of 0.1 ml of the aqueous MMC solution to the Difco albumin after treatment with guanidine hydrochloride resulted in a positive dithizon reaction in the dialysate. However, this reaction remained negative when 0.5 ml of aqueous MMC, 1.0 ml or 0.5 ml of the alcoholic MMC solution was added. The positive dithizon reaction in the first case seems to indicate that 1.0 ml of the aqueous MMC represented an excess of Hg-ions, leading to diffusion of unbound mercury through the membrane. A negative nitroprusside reaction indicated that all SH-groups were occupied. The results are summarized in Table II.

Minced preparations of skin, striated muscle and pancreas from calf were suspended in guanidine hydrochloride solution (25 gm in 50 ml solution), and 1.0 ml 0.1 —M KCN solution added. The suspensions were diluted with distilled water to a total volume of 200 ml and 10.50 and 75 ml aliquots were dialysed against distilled water after addition of various amounts of MMC (see Table II). The nitroprusside reaction was performed on the contents of the tubes after the dialysis. The dithizon reaction was performed on the dialysate. The results are shown in Table II.

Diffusion of mercury ions through the membrane seems to take place when MMC is added in excess in relation to the SH-groups present. The dithizon reaction in the dialysate was positive. Increasing the volume of the minced tissue preparations (increased total number of SH-groups available), as well as decreasing the concentration of mercury ions in the added reagent will then establish an equilibrium resulting in a negative dithizon reaction outside and a negative nitroprusside reaction inside the permeable membrane. In no case was the concentration of SH-groups in the minced tissues sufficient to give a positive nitroprusside reaction after the addition of MMC. These results show that in protein solutions and in minced tissues where SH-groups are present, addition of MMC leads to the formation of non-dialyzable mercury compounds.

3. In order to prove the specificity of the reaction of the mercury compound with the SH-groups, the latter were eliminated before the reaction was performed. For this purpose SH-blocking reagents were considered unsuitable, since little is known about the competing effect of mercury compounds in the equilibrium of this blocking reaction. However, iodine and hydrogen peroxide are known to oxidize the SH-groups to disulfide linkages, and such a reaction would be suitable for the following application. Therefore native and denatured egg albumin (Difco), as well as minced tissues, were treated with guanidine hydrochloride. A saturated aqueous iodine solution was added until the brown iodine colour remained stable. In another experiment instead of the iodine solution 1.0 ml of a 30 per cent solution of hydrogen peroxide was added. Various amounts of the MMC-reagent were then added, and the mixtures dialyzed against distilled water. Nitroprusside and dithizon reactions were then performed on the contents of the dialysis tubes and the dialysates. The results are shown in Table III.

If the treatment with iodine was incomplete, the addition of small amounts of MMC gave a negative dithizon reaction in the dialysate indicating that mercury ions were bound to remaining SH-groups. Experiments in which the SH-groups were eliminated by means of oxidation, addition of MMC resulted in the diffusion of unbound MMC into the dialysate. These results indicate that the presence of SH-groups was necessary for the binding of the mercury reagent. If dialysis against running tap water was continued for 3 days, no mercury could be detected in the albumin solutions, or in the minced tissues, acidified with hydrochloric acid. This was additional

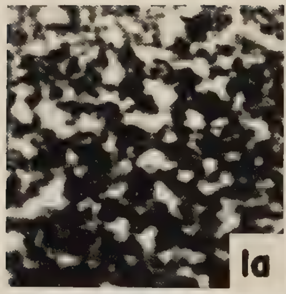
TABLE III

	Dithizon reaction in the dialysate after treatment with		Nitroprusside reaction in the tubes			
	J_2	H_2O_2	before		after	
			J_2	H_2O_2	J_2	H_2O_2
Egg albumin, native.	+	+	+	+	–	–
Egg albumin, Difco	+	+	+	+	–	–
Skin, calf, minced	+	+	+	+	–	–
Muscle, calf, minced	+	+	+	+	–	–
Pancreas, calf, minced . . .	+	+	+	+	–	–

evidence that the mercury compound had specific affinity to free SH-groups, and that the reagent was incapable of breaking disulfide bonds.

4. The nature of the bond between the mercury compound and the SH-groups was studied in the following experiments. Minced tissue and proteins containing sulfhydryl groups were treated with guanidine hydrochloride (nitroprusside reaction positive) and an excess of MMC was added. The mixtures were dialyzed against running tap water for 5 days, and then continued against 100 ml of distilled water for 12 hours, i.e. until all excess mercury was dialyzed from the tubes, as indicated by a negative dithizon reaction in the dialysate. The contents of the dialysis tubes were then acidified with hydrochloric acid, A positive dithizon reaction now indicated the presence of mercury still remaining in the tube. This suggested that the mercury compound was not attached to the proteins by mass action or by means of simple concentration.

In another experiment, 50 ml aliquots of mercury-containing protein solutions, dialysed for 5 days as described above, were then dialysed against solutions of 0.0001 M glutathione, 0.001 M cystein, and 7 per cent thioglycolic acid with additional 2 drops of 0.1 M KCN solution. After dialyzing for 48 hours, mercury was found to be present in the dialysate. Dialysis against the 0.1 M KCN alone did not result in any diffusion of mercury. Therefore it would appear that the labile and active SH-groups of the simple thiols had competed with the protein-bound SH-groups for the MMC. The mercaptides thus formed returned to the dialysate, and at the same time the proteins regained their SH-activities. The latter experiments have given evidence that the highly specific reaction between the MMC-reagent and the sulfhydryl groups is reversible.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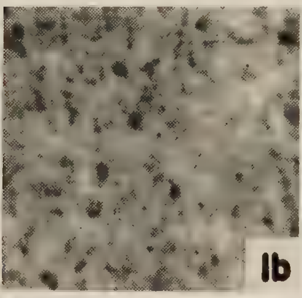
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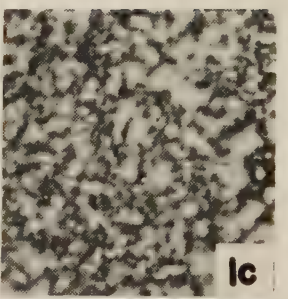
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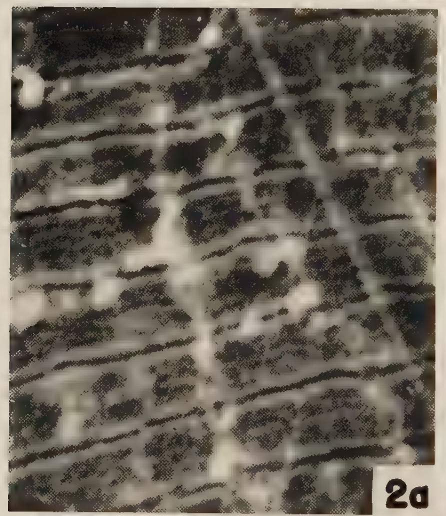
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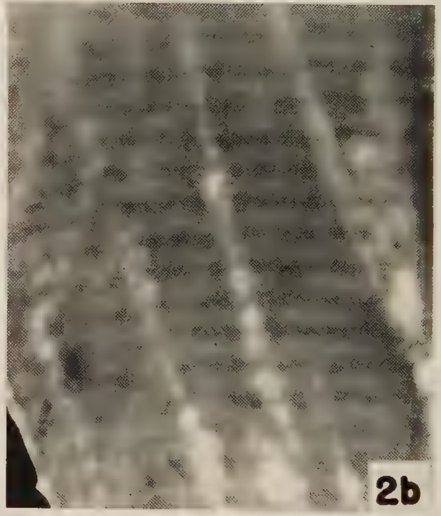
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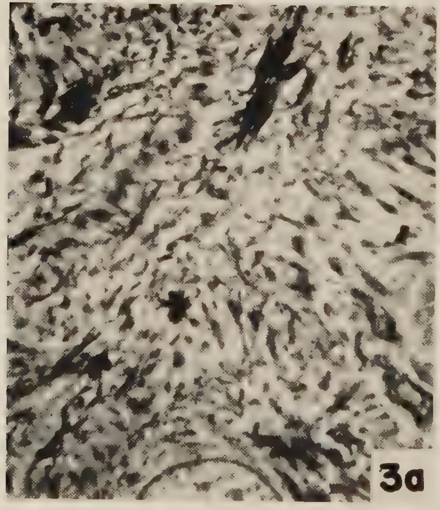
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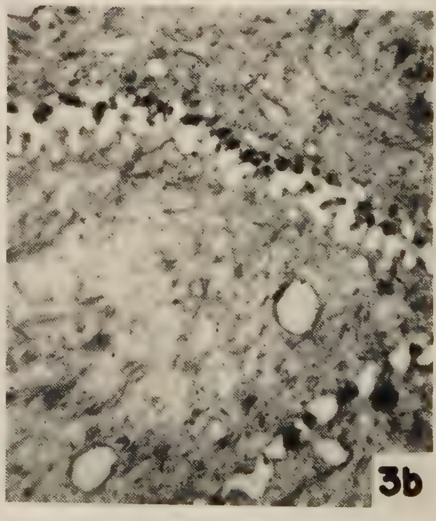
3b

APPLICATION IN ELECTRON MICROSCOPY

Deposits of mercury on the reaction sites of MMC with SH-groups would result in an increased electron scattering. To make its SH-groups available native egg albumin was incubated for 5 minutes with 5 ml guanidine hydrochloride at 37° C, followed by rapid cooling to 0° C. In some samples the SH-groups were then destroyed by oxidation with hydrogen peroxide. The samples were carefully washed, treated with saturated aqueous MMC-reagent and dialyzed against running tap water for 5 days. After dehydration in alcohol the specimens were embedded in methacrylate. Serial sections with an average thickness of about 0.1 μ were cut with a Spencer rotary microtome. The electron microscopical pictures are shown in Fig. 1 a–c. The electron scattering is much higher in the albumin preparation where available SH-groups have reacted with the mercury reagent (a) than in the untreated specimen (b) or in the MMC-treated preparation where the SH-groups had been destroyed by oxidation (c).

Protein-bound sulfhydryl groups are demonstrable in the striated muscular tissue by means of histochemical methods (e.g. 4). Small pieces of striated muscle from a mouse were directly submerged into a mono-ethanol-amino-phosphate buffer $[(HO \cdot CH_2 \cdot NH_2 \cdot NH_2)_2H_3PO_4]$ at pH 7.2 according to Thies and Kallinich (29). This new buffer was chosen because of its close biochemical relationship to biological substances such as kephalin, and was regarded to have a minimum of denaturating effect combined with suitable osmotic properties. The buffer was saturated with MMC and pieces of muscle were treated with the solution for 48 hours. Pieces of muscle adjacent to the above were fixed in osmium tetroxide in a veronal-buffer at pH 7.2. All specimens were dehydrated in alcohol and embedded in methacrylate. The electron pictures are shown in Fig. 2 a–b. In the muscular tissue treated with the mercury reagent, cross bands with high electron density can be seen (a). Also in the untreated muscular tissue, cross bands can be seen but these are faint, unsharp and hardly visible on the electron image of sections of corresponding thickness (b). The osmium-fixed preparations gave electron

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Fig. 1. Egg albumin, electron micrograph, ×8400. 0,1 μ sections of methacrylate-embedded material; a) after treatment with MMC; b) untreated specimen; c) treated with hydrogen peroxide prior to treatment with MMC.

Fig. 2. Striated muscle, mouse, ×8000; a) after treatment with MMC; b) untreated.

Fig. 3. Plantar epidermis, rat, ×5400; a) after treatment with MMC; b) fixed in buffered osmium tetroxide.

pictures corresponding to the well known electron histology of striated muscle (e.g. 5, 11, 16).

In epidermis the tonofibrils are generally assumed to be morphological elements involved in keratin production (for review see (14)). The oxidation of sulfhydryl groups in the stratum mucosum into disulfide linkages in the *stratum corneum* has been considered to be a fundamental process during keratinization. The presence of protein-bound SH-groups in the *stratum mucosum* has been established with chemical as well as with histochemical methods (e.g. 4, 14). The relation of the SH-groups to the tonofibrils is therefore of special interest. Fresh pieces of the plantar skin of the rat were treated with aqueous MMC for 30 minutes, rinsed in tap water for 1 hour, dehydrated in alcohol and embedded in methacrylate. Control pieces were fixed in osmium tetroxide in the usual way. A few pieces were also embedded after mere dehydration. The mercury-treated epidermis was distinguished by its fibrillar appearance (Fig. 3, a). The nodules of Bizzozero had about the same electron density as the intracellular fibrils. The untreated specimens showed no marked structures. In the osmium-fixed epidermis (Fig. 3, b) the fibrillar net was very faint but the nodules of Bizzozero showed a marked increased density. The fibrillar arrangement of the mercury deposits seems to indicate a close relationship between the localization of the SH-groups and the tonofibrils, thus supporting the assumed role of these elements during the process of keratinization.

DISCUSSION

Simple mercury compounds with the configuration R—Hg –X have great affinity for available, i.e., protein-bound, SH-groups. The reaction has a high specificity, and a reversible quantitative relationship exists between the SH-groups available and the number of molecules of the mercury compound. The investigation of the biochemical properties of the simple mercury compound, MMC, in relation to protein-bound SH-groups has confirmed these general facts.

Freshly minced tissues of striated muscle, skin and pancreas contained considerable amounts of protein-bound, available SH-groups, as indicated by a positive nitroprusside reaction. The masked SH-groups of native egg albumin are made easily available by the addition of guanidine hydrochloride. By addition of MMC to proteins or minced tissues with available SH-groups, a negative nitroprusside reaction indicates that a blocking of the SH-groups takes place. The mercury-containing protein molecules are non-dialyzable. An excess of the reagent, or a decreased volume of the SH-containing samples, results in diffusion of unbound mercury molecules through the membrane during dialysis. This indicates that a quantitative relationship exists between the mercury compound and the SH-groups available. KCN, which was added during the mincing procedure to prevent oxidation of the SH-groups, decreases the activity of mercury compounds (13). However, the very low concentration (0.1 molar) of the KCN-solution and the complete reaction of the mercury compound with the SH-groups, shows that the obstruction of the reaction by the presence of KCN is negligible. Neither did KCN prevent the diffusion of the unbound mercury reagent through the membrane during dialysis.

The specificity of the reaction between the MMC and the SH-groups is strongly supported by the fact that no mercury was attached to proteins when their SH-groups had been previously eliminated by oxidizing agents. Subsequent prolonged dialysis against running tap water resulted in completely eliminating the mercury from the dialysis tube. The same result was obtained with proteins, such as gelatin, where SH-groups are absent. Bonds between the mercury reagent and amino- or carboxyl groups of the tissue proteins cannot be excluded but the quantities are beyond the limit of detection and can be neglected. The CH_3-group of the reagent can be regarded as inert in this respect. The competing effect of thiols indicates that the reaction of mercury with the proteins is reversible and of the mercaptide type.

The application of the reagent in electron microscopy has revealed that the structures are better preserved in tissues treated with the reagent than in corresponding tissues which had only been dehydrated before the embedding. Thus MMC also serves as a fixative.

Mercury chloride, which is used as a constituent in various fixatives, gives bivalent reactions with sulfhydryl groups. Coupling of 2 SH-groups to one mercury-atom will give a SH/Hg ratio of 2:1. MMC, on the other hand, gives a monovalent reaction with a more favourable ratio of 1:1 and is therefore to be preferred.

In egg albumin preparations, as well as in tissues containing available SH-groups, the mercury reagent causes an increased electron scattering compared with the untreated specimens. Since the reaction between the reagent and the SH-groups has proved to be quite specific, the sites of mercury deposits seen on the electron image shows the distribution of the SH-groups in the tissue structures. This is supported by the fact that the increased electron scattering disappears when the SH-groups are destroyed by oxidizing agents prior to the treatment with the mercury reagent.

SUMMARY

Methyl-mercury-chloride is shown to have a high specificity for sulfhydryl groups in biological material and is introduced as a specific reagent ("electron stain") for the demonstration of these groups in electron microscopy. The reagent is applied on striated muscle and epidermis.

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BRIEF NOTES

THE FINE STRUCTURE OF THE NUCLEAR MEMBRANE IN THE LARVAL SALIVARY GLAND AND MIDGUT OF *CHIRONOMUS*

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In the course of electron microscopical investigations on the giant chromosomes of *Chironomus* (3) some interesting observations were made concerning the submicroscopic structure of the nuclear membrane. In view of the few studies so far made in this field these accidental observations seem to us worth while to be reported.

In 1950, Callan and Tomlin (5) had studied isolated nuclear membranes of the oocytes of *Triturus* and *Xenopus* by means of the electron microscope and found them to consist of two layers, a porous external one and a continuous internal one. Later, Bairati and Lehmann (2) as well as Harris and James (6) were able to demonstrate the same principal organisation for the nuclear membrane of *Amoeba proteus*, the porous layer being the internal one in this case. Recently, Anderson (1) postulated the existence of such a porous structure from biochemical data, a view which was criticized by Hogeboom and Schneider (7).[1]

In our material[2], i.e. sections of the salivary glands and the midgut of *Chironomus* larvae (mainly *C. tentans*), the nuclear membrane was again found to be porous in a regular pattern (Figs. 1–4). In the salivary glands, cross sections of the nuclear membrane show two sharply defined parallel layers enclosing a much less absorbing space between them. This compound layer system is completely perforated by numerous round pores, the brims of which are not pronounced as a rule. The average diameter of the pores is 500 Å, the spacing between the centers of the pores being 800–900 Å on the average. The overall thickness of the membrane is close to 300 Å, while the two layers themselves have 70 80 Å each. All figures given are based on at east 50 individual measurements. The homologous elements of the nuclear membrane n the cells of the midgut have dimensions of the same order of magnitude (cf. Table I). In contrast, however, to what is the rule in the salivary glands, the nuclear pores

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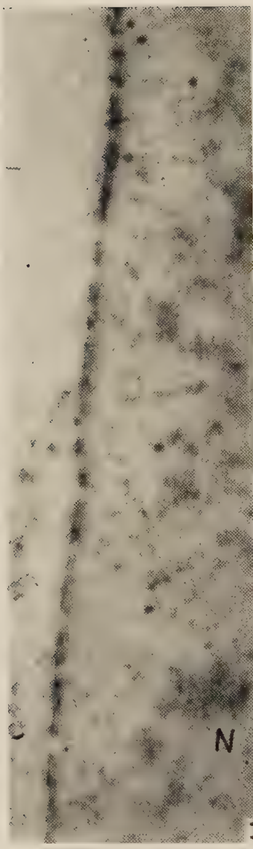
[1] In a paper of Brenner (4), brief reference is made to a study by Bovey on the nuclear membrane of *Chironomus*, quoted in a paper of Wilkins. The results of this work are not known to us.

[2] For methods of preparation see (1).



1

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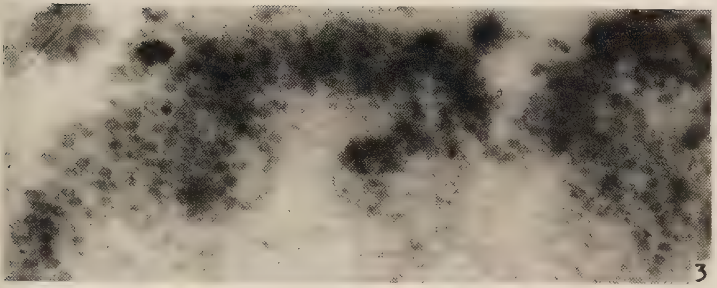
Fig. 1. Cross section of the membrane of a salivary gland nucleus of a *Chironomus* larva. N = nuclear content. 31500 ×.
Fig. 2. Slightly tangential cross section of the salivary nuclear membrane after 3 minutes treatment of the glands with a saturated solution of lanthanum acetate in distilled water before fixation in buffered OsO_4. Details in the text. 25200 ×.

in the midgut appear encircled by rings of heavily absorbing material. This phenomenon is very similar to what has been found in amphibian oocytes after OsO_4-fixation (3). Whether or not it is a fixation artefact (to which the midgut would then be subject more than the salivary glands) is not known. How fixation may influence the appearance of the nuclear membrane has been shown by the earlier workers and is demonstrated by Fig. 2, where the salivary glands had been pretreated with a solution of lanthanum acetate in distilled water: While the pores themselves are barely discernible, conspicuous granules have assembled upon the inside of the nuclear membrane, all or part of which possibly stem from the Balbiani rings of the chromosomes (1).

The observations reported here are in general agreement with the results obtained on other objects as regards the porousness of the nuclear membrane. However, the membrane of the giant nuclei of *Chironomus* larvae has no supporting continuous layer in addition to the porous one, which could be compared to those found in the



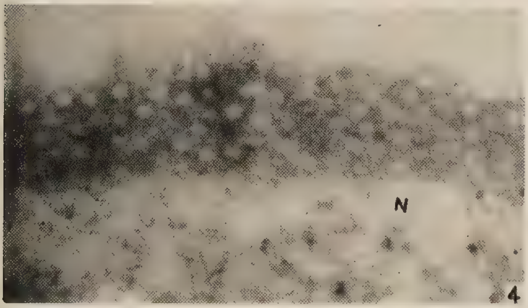


Fig. 3. Tangential section of the nuclear membrane in a cell of the larval midgut of *Chironomus*. 15750 ×.
Fig. 4. Tangential section of a salivary gland nuclear membrane. 31500 ×.

TABLE I

	Thickness of membrane	Distance of pore centers	Pore diameter
Triturus, Xenopus oocytes (5). .	450–500 Å	800–1 300 Å	300–500 Å
Amoeba proteus	3 000 Å	1 200 Å	800 Å
Chironomus sal. gland[1]	280 Å	800– 900 Å	500 Å
Chironomus midgut[1]	300 Å	800–1 000 Å	280 Å (with ring 350 Å)

giant nuclei of amphibian oocytes and *Amoeba proteus*, and it is much thinner than the latter. Thus, essential structural principle common to the observations on three widely divergent organisms is the porosity of the nuclear membrane whenever the nucleus is very large. If one considers the pores as places of high permeability, this principle is easily understood, for in giant nuclei the rate of transport through the membrane per unit area is necessarily higher than in nuclei (and cells) of normal size.[2]

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[1] Mean values of 50 measurements each.

[2] While this article was in press, B. Afzelius (Department of Anatomy, Karolinska Institutet) presented a paper at the 1954 meeting of the Scandinavian Society for Electron microscopy about fine structures in sea urchin eggs. He too showed porous nuclear membranes, but different from those described above. The later results will be published.

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Experimental Cell Research, **6**, *523–524* (*1954*)

ÜBER DIE KULTUR VON GEWEBEZELLEN *IN VITRO* AUF EINEM PLASMAFILM

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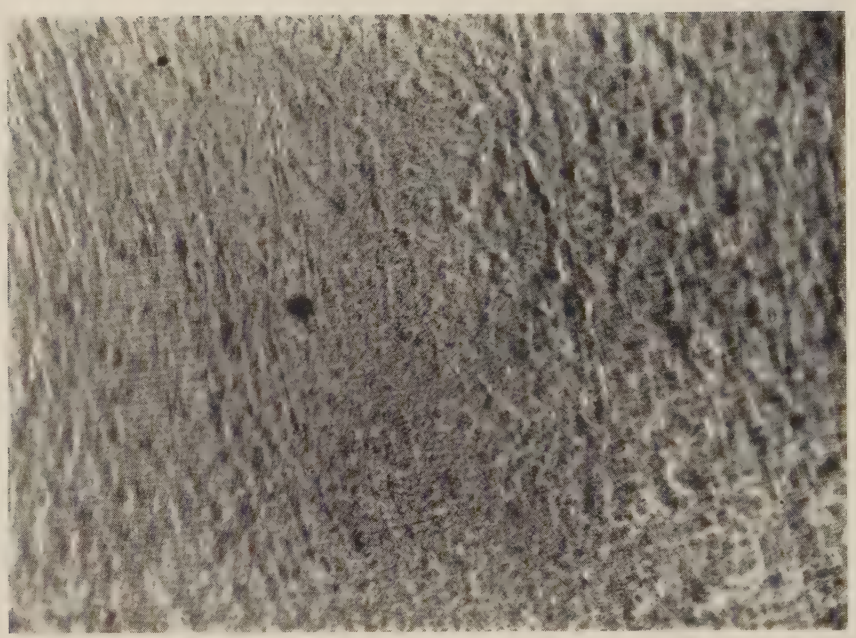
Eingegangen am 8. Januar, 1954

Herrn Professor Dr. Otto Hahn zum 75. Geburtstag gewidmet.

Während der Arbeit mit synthetischen Medien war es eine Zeit lang erwünscht, eine Gewebezüchtungstechnik anzuwenden, wo die Menge des dialysierten Plasmas auf ein Minimum herabgesetzt war. Die ,,roller-tube"-Technik, wie sie von G. O. Gey (3) und anderen angewendet wurde, konnte nicht benutzt werden, da es bei ihr nicht möglich ist, das ,,Wachstum" der Explantate planimetrisch in grossen Zügen zu verfolgen. Es wurde daher eine einfache Technik ausgearbeitet, wo die Gewebekulturen (embryonale Herzfibroblastzellen) auf einer Plasmahaut in Carrelflaschen gezüchtet werden.

Etwa 1 ml Hühnerplasma wird in eine Carrelsche Flasche eingeführt, und die Flasche wird rasch durch eine Flamme gezogen. Nach zehnminütigem Stehen wird das Plasma wieder herausgesaugt. In der Flasche verbleibt dann eine ganz dünne Plasmahaut (weniger als 1/15 mm dick). Die Gewebekultur wird auf diese angebracht, und nach etwa einer Viertelstunde wird die flüssige Phase hinzugefügt. Die Medien sollen Zimmertemperatur haben, da die Haut gegen Temperaturschwankungen empfindlich ist. Es wurden verschiedene Verdünnungen vom Plasma mit Tyrodelösung durchprobiert, von 1:1 bis 1:10. Es erwies sich, dass Verdünnungen bis hinauf zu 1:4 angewendet werden konnten, ohne dass eine wesentliche Gefahr für ein Zerreissen der Haut bestand. Häute aus dialysiertem Plasma erwiesen sich als ebenso beständig wie solche aus nicht dialysiertem Plasma. Dagegen konnte festgestellt werden, dass in den Kulturen, wo der flüssigen Phase Kompensationsstoffe für die hinausdialysierten Komponenten des Hühnerplasmas hinzugesetzt worden waren, ein Zerreissen der Haut viel häufiger eintrat, als in der Kontrollgruppe, in der die flüssige Phase aus 40 % Pferdeserum, 40 % Tyrodelösung und 20 % Embryonalextrakt bestand. Versuche, die Haut durch Zusatz von Heparin oder Sojabohnen Inhibitor zum Plasma zu stabilisieren, blieben ohne Erfolg. Das Letztere ermöglicht nach A. Fischer (2) die Züchtung von Geweben mit starker proteolytischer Aktivität auf homologem Medium.

Mit Hilfe dieser einfachen Technik erzielt man schöne Kulturen, die aus 1–2 Zellagen bestehen. Die Ausbreitungsgeschwindigkeit ist folglich grösser als bei der Züchtung im Plasmagerinnsel. Wenn die flüssige Phase ausgewechselt wird, halten sich die Kulturen ziemlich lange, doch ist die Haut nach etwa einem Monat teilweise verdaut, und es tritt eine Unzahl isolierter Zellen auf, von denen viele Rundzellen sind. Obwohl es möglich ist, die Kultur herauszukratzen und sie weiterzuführen, eignet sich die Methode nicht zur Züchtung von Passagenkulturen. Nach



etwa zehn Tagen wird selbst in den Kulturen, wo die flüssige Phase proteinfrei ist (das Protein ist durch eine Mischung von Aminosäuren ersetzt in dem Verhältnis, wie sie im Fibrinogen vorkommen), eine Anhäufung von Fett auftreten. Dies bringt die Untersuchungen von Å. Bohus Jensen über die Fettanhäufung in Gewebekulturen in Erinnerung: In Zellophankulturen mit proteinfreiem Medium wurde keine Bildung von Fettröpfchen im Zytoplasma beobachtet (1). Beim Zusatz reiner Proteinstoffe trat dagegen eine Fettanhäufung nach etwa 10 Tagen auf (nicht veröffentlicht). Man kann also mit einiger Wahrscheinlichkeit schliessen, dass die Plasmahaut ausser ihrer Funktion als Stützstruktur auch als Nahrung dient. Die Morphologie der Plasmahautkulturen ist einer mikroskopischen Untersuchung erheblich leichter zugänglich als es bei den üblichen Verfahren der Fall ist, wo die Kultur in einem Plasmagerinnsel eingebettet wird. Beim Fixieren und Färben in der Flasche werden gute Resultate erhalten, da ein Überfärben des Plasmas vermieden wird. Die Methode wird sich besonders gut eignen für pharmakologische Studien *in vitro* und für Untersuchungen über die wachstumshemmende und wachstumsfördernde Wirkung gegebener Stoffe auf Gewebezellen *in vitro*.

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Experimental Cell Research, **6**, *525–526* (*1954*)

APPOSITION TECHNIQUE WITH STRIPPING FILM FOR AUTORADIOGRAPHY

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We have made several attempts to use very thin emulsions for autoradiography in such a way that the film is separated from the histological section after the exposure (apposition technique). Of several attempts, the following technique seems to be the best one, see Fig. 1.

The histological specimen and the stripping film (Kodak Autoradiographic Plate) are mounted on two separate slides of methylmethacrylate. Dust-free handling is important. The sensitive side of the film must be turned outwards. During the exposure the slides are brought in very close contact by pressure. After exposure, suitable processing as described elsewhere (1) is undertaken with the stripping film, and any histological staining can be chosen for the histological section. Perfect alignment of autoradiograph and section can be obtained in the following way. Thin lines are drawn on the object slide. These are copied optically on the film in undisturbed position (the specimen shielded) before the processing.

The resolution of this technique is estimated to about 8 μ. Figs. 2 and 3 exemplify a result.

The advantages of this technique are: 1) The geometry is defined, enabling quantitative evaluation according to the principles given earlier (2). 2) The optical alignment is sufficiently good, and is determined by the optical resolution of the emulsion. This is always better than the autoradiographic resolution. 3) Any histological staining may be chosen. 4) the photographic processing is uninfluenced by the section. 5) Microdensitometry can be performed without optical disturbances from the section. 6) Serial autoradiographs can be made of one section. This is important in activation analysis of histological sections and in work with multiple tracers.

The disadvantages are: 1) The resolution is not as good as in ordinary stripping film technique. 2) The stripping film has a great tendency to loosen from the metha-

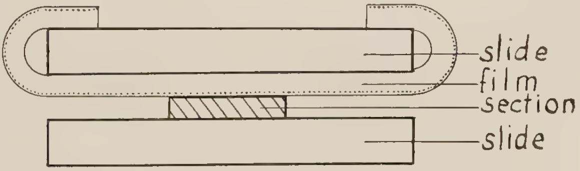
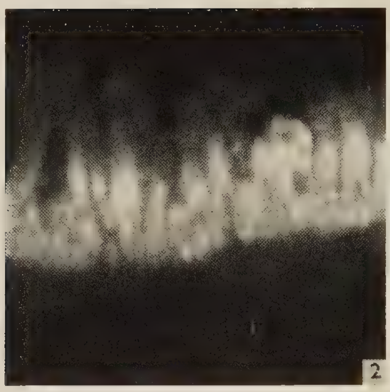


Fig. 1. The principle of the apposition of film and histological section is showed.



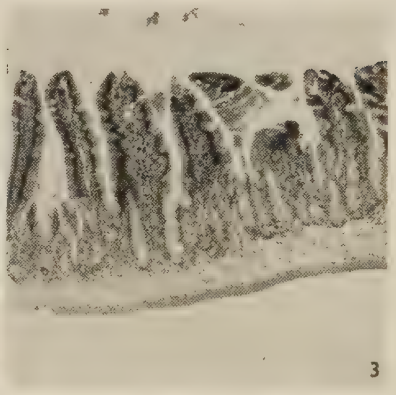


Fig. 2. Autoradiograph (51 ×) of small intestine of the mouse after injection of radiophosphate (P^{32}). Villi, separated by a distance of 8 μ (on the figure about 0.4 mm), are clearly separated, which illustrates the resolution of this technique.

Fig. 3. The corresponding part of the histological section (51 ×) stained with haemalum-eosin.

crylate slide, which may spoil the autoradiograph totally. 3) The interpretation of the pictures in the microscope after alignment is difficult at high magnification, because the preparation is surrounded by slides on both sides.

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UPTAKE OF RADIOSULPHATE AND RADIOPHOSPHATE IN VARIOUS TISSUES OF NORMAL AND SCORBUTIC GUINEA PIGS

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The rate of collagen formation is diminished in scurvy (2, 4). The effect of C-avitaminosis on the mucopolysaccharides in the ground substance of the connective tissue has not yet been completely clarified but histochemical investigations tend to indicate a reduced formation of polysaccharides (5, 6), eventually with an accumulation of products of a lower degree of polymerization (3, 7). Observations on the fate of radiosulphate in animal organisms seem to support the view that the uptake in the connective tissue and cartilage is mainly due to an incorporation in the sulphomucopolysaccharides (1). The metabolism of these substances can thus preferably be studied with labelled sulphate. Reddi and Norström (8) recently found that the incorporation of radio-sulphate is considerably reduced in chondroitin sulphuric acid, in scorbutic guinea pigs. The aim of the present investigation is to study the uptake of sulphate in scurvy and to relate this uptake to the specific scorbutic bone changes. A simultaneous determination of the uptake of radiophosphate to some extent gives a comparison as to the effect of C-avitaminosis on the mineral part of the bone tissue.

Method. Young female guinea pigs, weighing about 200 g each, were given a modified Höjer scorbutogenous diet (4). Within three weeks they had developed a typical clinical scurvy and did not survive five weeks. Control animals which were given an adequate addition of C-vitamin showed normal weight increase and vitality. Each individual in one group of animals was given an injection of carrier-free S^{35}-labelled sodium sulphate. The dose was 3.0 mc/kg in 0.9 per cent saline with 0.02 per cent sodium sulphate as a carrier. Another group received P^{32}-labelled carrier-free orthophosphate in a dose of 0.5 mc/kg in 0.9 per cent saline at pH 6.7 containing 0.01 per cent phosphate. All injections were given intraperitoneally. The animals were killed 48 hours after the injections. Small portions (approx. $2 \times 10 \times 10$ mm) of the various organs were excised. Blood samples were also taken. The sections were dried for at least 48 hrs. at about 50°C, and repeated weighing revealed that they had attained constant weight. The radioactivity was measured in a standard way all through the experiment. The activity was corrected for background and decay and was expressed as counts/min./mg wet tissue.

Results. The table discloses that, with the exception of cartilage, the radiosulphate activity in various organs of the scorbutic animals was only about one third of the activity in normal animals' tissues. This difference is statistically significant. Cartilage showed the highest sulphate uptake but also the greatest inherent variations. In cartilage from scorbutic animals the activity was slightly lower than in the controls but this decrease can not be regarded as significant. No significant difference

TABLE I

Activity of radioactive samples.
Counts/min./mg wet tissue.

Organs	Radiosulphate				Radiophosphate			
	Normal animals n=4	Scorbutic animals n=6	Difference of means	Student's t	Normal animals n=6	Scorbutic animals n=9	Difference of means	Stu- dent's
Blood . .	0.52 ± 0.06	0.16 ± 0.03	0.36 ± 0.06	6.10***	9.0 ± 0.5	8.3 ± 10.8	0.7 ± 1.1	0.68
Liver . .	1.06 ± 0.17	0.26 ± 0.06	0.80 ± 0.15	5.12***	62.9 ± 3.7	74.7 ± 7.3	11.8 ± 9.6	1.23
Spleen . .	1.31 ± 0.17	0.45 ± 0.06	0.86 ± 0.15	5.54***	78.6 ± 6.5	67.8 ± 8.4	10.8 ± 11.6	0.93
Brain . .	0.20 ± 0.03	0.05 ± 0.01	0.15 ± 0.02	6.86***	6.9 ± 0.5	7.6 ± 0.8	0.7 ± 1.0	0.68
Kidney .	0.94 ± 0.13	0.33 ± 0.06	0.61 ± 0.13	4.77**	47.2 ± 5.5	56.9 ± 7.2	9.7 ± 9.9	0.97
Skin . . .	0.58 ± 0.05	0.18 ± 0.02	0.40 ± 0.05	8.35***	15.9 ± 2.2	13.8 ± 1.1	2.1 ± 2.2	0.95
Muscle . .	0.24 ± 0.03	0.09 ± 0.02	0.15 ± 0.04	4.34**	19.9 ± 2.6	20.1 ± 1.0	0.2 ± 2.4	0.09
Cartilage	6.50 ± 2.18	5.17 ± 1.39	1.33 ± 2.45	0.34	52.2 ± 11.7	64.2 ± 6.4	12.0 ± 12.3	0.98
Bone . .	1.51 ± 0.27	0.55 ± 0.08	0.96 ± 0.24	4.06**	142.4 + 17.1	92.6 ± 17.0	49.8 + 17.0	2.93*

between the radiophosphate uptake in normal and scorbutic animals could be established. In bone, however, the radiophosphate uptake in the scorbutic animals showed a probably significant decrease of about one third.

Discussion. A considerable decrease in the uptake of radioactive sulphate in the various organs of scorbutic animals was found. Cartilage, however, showed no significant diminution in the uptake of sulphate, but the manifest tendency is in agreemen with earlier observations (8). At present it is not possible to give an exact explanatio of these findings. If present views on the fate of radiosulphate in the animal organism are valid, it is justifiable to assume that our results at least partially reflect a diminished sulphomucopolysaccharide formation. If that is the case, vitamin C is of importance for the synthesis of these polysaccharides, as well as for the formation of collagen. It would then be tempting to assume that the sulphomucopolysaccharides are in some way involved in the synthesis of the fibrillar proteins. On the other hand there was no apparent, essential change in the metabolism of phosphate. Bone alone showed appreciable lowering of the radiophosphate uptake in the scorbutic animals. It is not yet possible to state whether this decrease—or the decrease in radiosulphate uptake—is due to a reduced incorporation in the organic and/or mineral parts of bone.

The experiments proceed and a full account of the work will be published elsewhere.

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AN APPARATUS FOR THE MEASUREMENT OF THE LIGHT DISTRIBUTION AROUND MICROSCOPICAL OBJECTS

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In the ultra-microspectrophotometer for high resolution the absorption spectra of biological objects can be determined by utilizing the entire resolving power of the microscope. In order quantitatively to determine the absorbing substances in an object from this absorption curve the object and the measuring instruments must meet a series of conditions. For a more detailed treatment of these factors see Caspersson (1, 2). One of the factors of the greatest importance for the practical work is the requirement that the measured loss of light should be conditional only on the real absorption in the specimen. For this reason the optical system, in the first place the aperture of the condensor and of the objective, must meet certain special conditions. If the objective aperture is too small or the condensor aperture too large there will be a loss of light in the optical system, and thus part of the light leaving the object will not take part in forming the image. In the practical work on the variety of biological preparations this factor is of such dominating importance for all microspectrographic work that it must be made a condition for every curve analysis that no errors should appear owing to this factor.

The most adequate method for achieving this is to determine the light distribution around the illuminated specimen, and from this distribution curve to calculate the minimum value of the permissible objective aperture and the corresponding maximum value of the condensor aperture. At this Institute a simple apparatus, constructed by L. Larsson, has been used for establishing such light distribution curves in the visible spectral range for certain critical angles. This technique is sufficiently accurate for many types of biological objects (the apparatus is described in (3)). In this apparatus the object and the illuminating system were rotated around an axis that was perpendicular to the optical axis and went through the object. The light distribution curves were determined by means of a stationary photocell.

The following is the description of an apparatus which is used for more accurate measurements in regions of different wave lengths, in wide intensity intervals, and in an extensive angle-range. It is partly intended for determining the objective and condensor aperture necessary for objects in general, partly for experiments with different types of model substances. It is further equipped for making such exact measurements of light distribution that these can, if required, be used for calculating correction factors for spectrophotometric objects refracting light so intensely that the differences in the refractive index in these objects cannot be eliminated in the usual way, in which case there would be a certain error in the measurement of the light absorption itself.

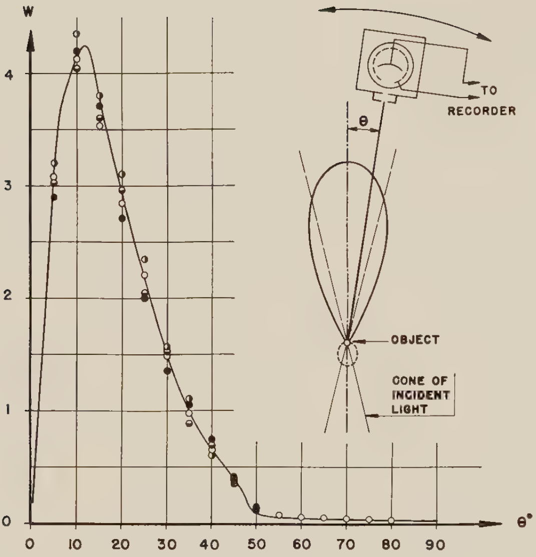

Fig. 1. Light distribution around a cylindrical model object. The principle of the measurements is inserted.

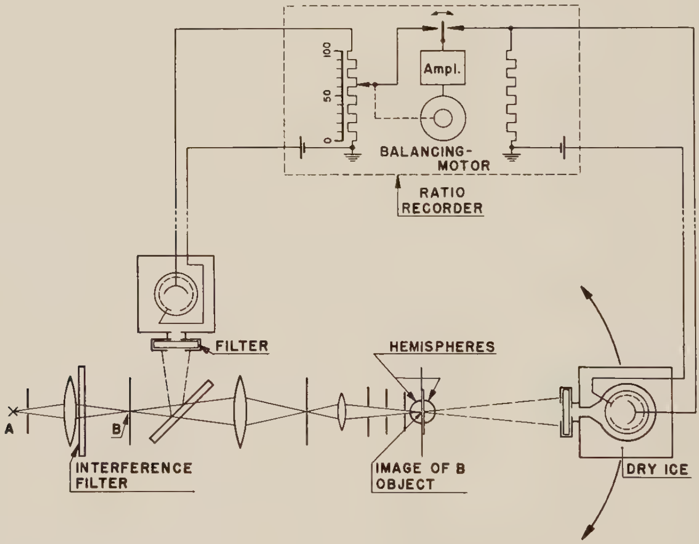
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Fig. 2. Diagram of the instrument. A is the source of light or the exit slit of the monochromator, if used. B is a small circular hole, which is projected into the specimen.

Fig. 1 shows the light distribution around a model object. The incident light energy will, on the one hand, be absorbed by the specimen and, on the other, be scattered at a greater solid angle than that of the incident ray, owing to refraction, reflection and diffraction. In most biological objects the reflection component (which is dotted in Figure 1) can be suppressed by using suitable preparation methods. The main interest is centred in the determination of the light energy distribution behind the object. The figure also indicates the principle of using a movable photocell to determine the curve of distribution.

In the instrument under discussion (Fig. 2) a small light spot is projected on to the specimen. The light leaving the specimen is taken up by a photomultiplier, which is moved around the specimen. The mechanical arrangements are such that the optical axis can be placed either horizontally or vertically in order that different sources of light may be used, e.g. concentrated arc lamps, Hg lamps or larger illuminating devices, such as spark gaps with monochromators for ultraviolet. Either monochromators or suitable filters may be used for selecting wave lengths. The diameter of the light spot in the specimen can be made to vary between a few micra and some millimetres. The optical system makes it possible both to determine the light distribution curve using parallel light and to measure the distribution of energy by means of a system closely imitating the one used in the spectrophotometer.

The photomultipliers used are two RCA 1P 21, one of which is used as a reference cell. From an amplifier one gets the quotient of the intensities of the two photomultipliers; thus the influence of the variations in the source of light is eliminated. The values obtained can be registered by a recorder; the light distribution curve can thus, if required, be recorded automatically by rotating the photomultiplier around the specimen, synchronously with the movement of the paper. The variations of the dark current are reduced by cooling the photomultiplier with dry ice. Owing to the enormous differences in intensity in the different directions diaphragms and filters with a known, high light-absorption are used in certain directions.

For the correct measurement of the light distribution a scattered light beam must neither be reflected nor refracted at the surfaces of the slide or the cover glass, and it must proceed undisturbed from the specimen to the photo-multiplier. This requirement is met by placing the object (with slide and cover glass) between two nonabsorbent hemispheres having the same refractive index as the slide and the cover glass and having its centre in the light spot in the object.

In order to test the instrument objects have been examined, which have such simple geometrical shapes that the theoretical light distribution curve can be calculated. The calculated curve of a cylindrical glass rod is shown in Fig. 1. The relative refractive index is 1.14, which would be extremely high for a biological object. The surface under the curve constitutes the amount of energy taken up by the different apertures of the objectives. It should be observed that less than 1 per cent of the total energy is located outside a cone with a top angle of 2 × 50°. In addition the graph shows the measuring values of a number of glass rods. Since the shape of the glass rods is not quite perfect, there are slight deviations from the theoretical curve; still there is good agreement between the theoretical curve and the measuring values.

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Experimental Cell Research, **6**, *532 534 (1954)*

THE ACTION OF TRYPSIN AND CHYMOTRYPSIN ON THE SEA URCHIN EGG

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Runnström *et al.* (7) observed that treatment of the unfertilized sea urchin egg with trypsin prevents the formation of a fertilization membrane after subsequent insemination. In a number of papers (1, 4, 8, 10) the properties of the surface layers of the sea urchin egg have been extensively investigated. The use of trypsin has become an important method to examine the behavior of the cortical material. For further literature see (6).

In the eggs of *Echinocardium cordatum*, Runnström (5) found that the vitelline membrane was elevated upon the addition of crystalline trypsin in low concentrations. Recently, Moore (3) made the same observation in the eggs of *Dendraster excentricus*. He also found that chymotrypsin was unable to induce membrane formation.

When investigating the re-fertilizability of the sea urchin egg it was found that trypsin and chymotrypsin had different effects (2). It was also observed that a crude preparation of trypsin (Merck, Darmstadt), which probably contained several other enzymes, rendered the eggs more susceptible than usual to polyspermic fertilization.

Material and Methods. Observations were made on a number of species found in the gulf of Naples: *Paracentrotus lividus*, *Psammechinus microtuberculatus*, and *Sphaerechinus granularis*. Similar experiments were also performed on the Swedish west coast with *Psammechinus miliaris* (S-form) and *Echinocardium cordatum*.

The eggs were pretreated with the enzyme; thereafter they were washed in sea water and subsequently inseminated. The cultures were examined with a phase-contrast microscope before the third cell-division when the polyspermic eggs are easily counted. The time of pretreatment was 10 minutes and the concentration of enzyme was chosen to inhibit the formation of a fertilization membrane in 95-100 per cent of the eggs. It is important to have a concentration as low as possible in order to avoid injury to the eggs. Thus the concentration of crystalline trypsin (Armour Co.) was 0.001 per cent, of crystalline chymotrypsin (Worthington Biochem. Lab.) 0.0006 per cent and that of crude trypsin (Merck, Darmstadt) 0.01 per cent.

Results. Pretreatment with crystalline trypsin in low concentrations (0.001–0.01 per cent) tends to induce polyspermy. The hyaline layer becomes slightly swollen and in higher concentrations the blastomeres begin to separate. However, the correct dosage of crystalline trypsin is rather difficult, since higher concentrations give marked polyspermy (2).

Chymotrypsin was found to have a weaker action on the eggs although the removal of the fertilization membrane occurs in very low concentrations. After pretreatment with chymotrypsin only a few eggs became polyspermic even if the concentrations were high. The hyaline layer seemed not to be damaged.

The Merck-trypsin was found to damage the mechanism preventing polyspermic insemination. As a rule heavy polyspermy occurred (25–100 per cent) in the lowest concentration ensuring 100 per cent membrane-free eggs. It often happened that every egg was entered by more than five spermatozoa; consequently the cytoplasmic division was stopped at an early stage. If more enzyme was used the hyaline layer became very swollen and finally dissolved, resulting in separated blastomeres. The proteolytic activity of Merck-trypsin was found to be more than 20 times weaker than that of crystalline trypsin.[1]

Several experiments (about 75) were carried out in which pretreatment with Merck-trypsin and chymotrypsin were combined. Each enzyme was allowed to act 10 minutes on the eggs. These were washed with sea water before the treatment with the second enzyme was applied. The concentration of crystalline chymotrypsin was 0.0006 per cent and that of Merck-trypsin 0.01 per cent. In the following table some results are shown from two experiments with *Paracentrotus lividus*.

TABLE I

Eggs pretreated										Control
10 minutes with chymotrypsin; then inseminated		10 minutes with chymotrypsin; inseminated. After 3 minutes a 10 minutes exposure to Merck-trypsin; then re-inseminated		10 minutes with chymotrypsin; then 10 minutes with Merck-trypsin; inseminated		10 minutes with Merck-trypsin; then inseminated		10 minutes with Merck-trypsin; then 10 minutes with chymotrypsin; inseminated		Eggs untreated; inseminated
Eggs fertilized per cent	Polyspermic eggs per cent	Eggs fertilized per cent	Polyspermic eggs per cent	Eggs fertilized per cent	Polyspermic eggs per cent	Eggs fertilized per cent	Polyspermic eggs per cent	Eggs fertilized per cent	Polyspermic eggs per cent	Eggs fertilized per cent
100	1.8	99	2.5	100	6.0	97	26	96	0.8	100
100	7.0	99	7.0	74	3.0	100	73	77	0.5	98

The number of spermatozoa used in each insemination was 2.6×10^6/ml. Sperm and eggs were allowed to interact for 3 minutes before the second treatment with chymo- or Merck-trypsin. In every culture 200–250 eggs were counted.

Discussion. The morphological changes of the hyaline layer obtained after treatment with Merck-trypsin are very clear. The high percentages of polyspermic eggs seem to justify the interpretation that the visible changes of the hyaline layer may cause the loss of the block to polyspermy.

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[1] The determination was kindly performed by Dr. G. Lundblad using a viscosimetric method with gelatine as substrate.

Eggs treated with clupein or lysozyme also show a marked tendency to polyspermy (2, 9). Clupein as well as lysozyme precipitates the cortical granules; this may indicate that these granules take part in preventing polyspermy. However, clupein and lysozyme may also act on other structures of the egg surface and the effect of these substances is perhaps due to changes in both the egg surface and the cortical granules.

As seen from the results, chymotrypsin has the ability to reverse and counteract the action of Merck-trypsin. The effect of chymotrypsin favors the view that some substance is released that restores the block to polyspermy. Whether this factor is concerned with the cortical granules or not remains unsettled, since morphological studies have not revealed any clear differences.

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Experimental Cell Research, **6**, *535–536 (1954)*

BIOPHYSICAL STUDIES ON BONE TISSUE

IX. X-RAY DIFFRACTION STUDIES ON BONE TISSUE FROM OSTEOGENIC SARCOMA

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In a previous paper, a report was given of a biophysical study of the neoplastic bone tissue obtained from 16 cases of osteogenic sarcoma (1). The characteristics of the tumor tissue have been investigated using X-ray-absorption measurements, polarized light and *in vitro* ionic exchange reactions. In order to obtain further information concerning the properties of the tumor tissue on the ultrastructural level, an X-ray diffraction study has been performed on this material and the results are given in this communication.

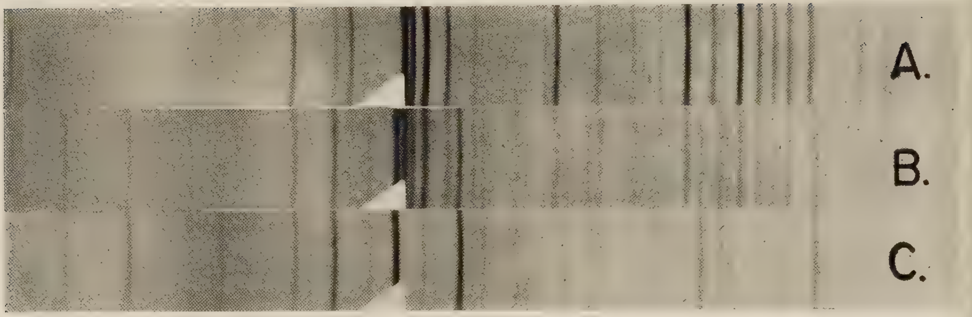
Material and methods. Sixteen cases of osteogenic sarcoma were studied. The parts of the infiltrating sarcomatous tissue selected for this investigation were situated immediately adjacent to the tissues used for histological and histochemical investigations which were reported in a previous publication (1). Thin sections of the tumors were cut with a rotating saw and were either ground to a thickness of 100–200 μ or heated for one hour at a temperature of 900° C. The powders obtained after heating were put into thin walled glass capillaries with a diameter of 0.2–0.3 mm.

A Siemens Crystalloflex II apparatus with copper target served as radiation source and a 0.02 mm Ni-filter was used. The diffractograms were obtained either with a 57.3 mm Siemens cylinder camera or for precision measurements with a 190 mm Hilger cylinder camera. The diffraction patterns were registered on Ilfex X-ray film and processed in Kodak D 19b developer.

Results and comments. In the cases where histologic examination of the immediately adjacent tumor tissue had revealed mineralized tissue, a diffraction pattern characteristic of normal bone tissue could be demonstrated. However, in a few cases reflections from components other than calcium phosphates also were detected. These reflections were evidently due to contaminations from the fixing solutions. Only those parts of the tumor tissue which showed presence of bone salts were selected for a thorough analysis.

In the X-ray diffraction pattern from unfixed or fixed normal bone tissue the reflections are rather diffuse. Apparently this is due to the small size of the crystallites. With the aid of low-angle X-ray diffraction, the size of the crystallites in normal bone has been calculated to be about 75 × 210 Å (2).

The diffractograms from the mineralized tissue in osteogenic sarcoma showed diffuse reflections of the same appearance as does normal bone tissue. This indicated that the mineralized phase of the tumor tissue had crystallites of about the same size as in normal bone. Analogous to diffraction patterns from normal bone there was a marked sharpening of the reflections from tumor tissue after heating to 900° C for one hour, depending on an increase in size of the crystallites. This treatment enabled accurate measurements on the diffractograms and the size and structure of



A. X-ray diffraction pattern of heated tumor tissue from osteogenic sarcoma showing the same apatite pattern as that of normal bone.
B. Same as A, but reflections from a substance closely related to whitlockite (β-tricalcium phosphate) is superimposed on the apatite pattern.
C. X-ray diffractogram from a synthetic β-tricalcium phosphate with the same lattice constants as whitlockite.

the unit cell could be derived. In order to detect differences in the lattice constants of the apatite between normal bone and osteogenic sarcoma, the diffractograms were registered in the 190 mm Hilger cylinder camera which gave a high resolution.

The parts of the 16 osteogenic sarcoma which in the preliminary investigation were shown to contain apatite all gave diffraction patterns which after measurements showed values in accordance with those from normal bone. Interpreted on a hexagonal unit cell the axes were calculated to $a = 9.41$ Å and $c = 6.88$ Å. In four cases, reflections from a substance closely related to β-tricalcium phosphate were superimposed on the apatite pattern. Though the pattern had the same characteristics as that from pure β-tricalcium phosphate the axes of the unit cell were somewhat shorter. Measurements showed the axes to be $a = 10.3$ Å and $c = 37.0$ Å. These values were in good agreement with those given for the mineral whitlockite which mainly consists of β-tricalcium phosphate. One sample of this mineral containing 1.6 per cent magnesium (substituting calcium) was investigated by Frondel (3) and he gave the axes to $a = 10.3$ Å and $c = 36.9$ Å. A calcium phosphate precipitate obtained at pH 7 from solutions containing 0.005 M Ca, 0.003 M P and 0.0037 M Mg gave, after heating to 900° C for one hour, a diffraction pattern from which the same inter-planar spacings as those of whitlockite could be calculated. Reflections from this substance have not been detected in the non-heated specimens and it is thus probable that it was formed during the heating procedure. A possible explanation is that phosphate and magnesium in the tissue might have reacted with the apatite, and those tumours which showed the whitlockite-pattern revealed in general an abundance of organic substances, that is, they were highly cellular and mineralization was present only in small areas.

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Experimental Cell Research, **6**, *537–539 (1954)*

ABSORPTION SPECTROPHOTOMETRY WITH EXTREMELY SOFT X-RAYS FOR THE QUANTITATIVE DETERMINATION OF THE DRY WEIGHT OF CYTOLOGICAL STRUCTURES

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FROM measurements of the absorption of soft X-rays in thin sections (5–10 μ) of biological objects, the total dry weight of different structures can be determined *in situ* (7). With continuous X-radiation, generated at 3000 volts and filtered through 9 μ aluminium, a microradiogram of the biological sample and a reference system, consisting of a step wedge of nitrocellulose foils, is registered on a fine-grained photographic emulsion, which is in close contact with the specimen. The densities in the absorption picture of the sample and of the reference wedge can be determined photometrically on photomicrographs, but direct photometry in the enlarged image of the microradiogram gives more accurate results. Photometer equipment for this purpose has been constructed (8, 1). The different sources of errors in the original method have been discussed (11, 12), and new apparatus and modified technical procedures have been published (3, 4).

In order to obtain a higher resolution and accuracy, the author has modified the X-ray weighing procedure. New equipment has been constructed to utilize a wave length range of X-rays softer than those used before. These extremely soft X-rays are strongly absorbed even in very thin sections of biological samples. The great contrast obtained in these microradiograms allows the determination of smaller differences in dry weight than has previously been possible. A high-vacuum X-ray microspectrograph has been modified so that microradiograms can be registered with about 1000 volts across the X-ray tube. With the electrostatic focusing system grounded (zero potential) the blurring in the microradiograms is about 0.2 μ for tissue sections about 5 μ thick. With negative voltage applied to the focusing system, the blurring can be reduced still more.

Certain emulsion numbers of the Kodak Maximum Resolution Plates have a higher resolution than the Lippmann emulsions now available, and have a more even emulsion layer. A number of different developers have been tested, and the most satisfactory results were obtained with Kodak D 158, diluted with two parts of distilled water, with a developing time of 10 minutes at a temperature of 18.0°C. This fine-grain development gives a rather low gamma value, but this disadvantage is overcome by the strong absorption of the extremely soft X-rays in the specimen. It is difficult to state exactly the linear resolution in the microradiograms as it depends on several factors, such as the graininess of the film emulsion, the density, and the differences in dry weight. For an average biological material, the resolution obtained by the author varies between 0.6 and 0.8 μ (see Fig. 1). For quantitative

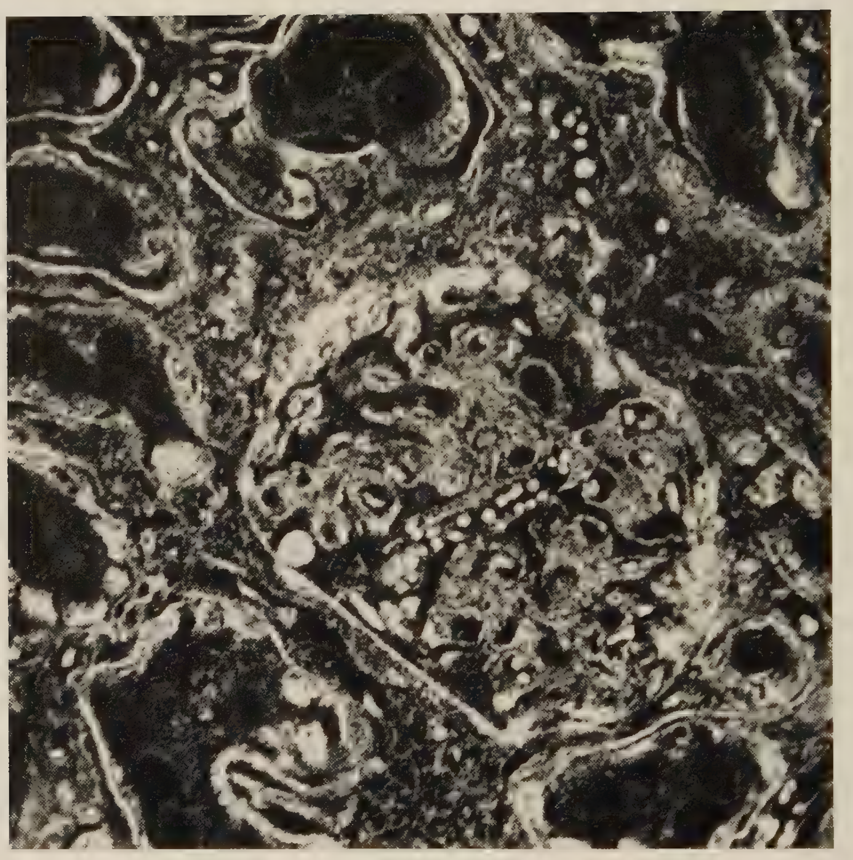

Fig. 1. Microradiogram of a 5 μ section of renal parenchyma from a case of chronic glomerulonephritis. Secondary magnification 400 ×. The light areas correspond to a high concentration of dry weight. A thickened Bowman's capsule and individual capillaries are seen in a glomerulus.

determinations, the smallest area to be measured depends on the specimen and on the accuracy desired. An area smaller than 2 μ^2 should rarely be measured.

In the original method of making the reference system, the standard error was ±11 per cent (11), depending on the difficulty of making even nitrocellulose foils on a water surface. With more elaborate methods of making the foils on water (2, 9), and interferometric methods of determining the thickness of the foils (10, 6), the accuracy is higher. Clemmons and Webster (5) used dyed Parlodion foils made on glass surfaces and measured the light absorption in the dyed wedge. Knowing the concentration of the dye and the density of the foil, the weight per unit area could be determined.

Experiments have shown that nitrocellulose foils made on a glass surface are much more even than those made on water. Therefore, the author now makes the foils for the reference system on glass slides, but no dye is used. Two areas of known size are accurately cut and weighed on a torsion balance, and the foils used for the reference wedge are cut from the area between the weighed parts. From a series of duplicates, it was found that the weight per unit area could be determined from the mean of these two determinations with a standard error of ±1.7 per cent. The photometry of the microradiograms reveals a very uniform thickness of the foils used as a reference system.

With this new X-ray equipment, improved technical procedures, and direct photometry of the microradiograms (registered on Kodak Maximum Resolution Plates), the dry weight per unit area in sections of squamous epithelium has been determined with a standard error of about ±5 per cent (the systematic error not included). To obtain the weight per unit volume, the thickness of the section must be determined, which involves a comparatively large error. Therefore weights per unit area and relative values should be used as far as possible. It must also be stressed that the standard error must be determined for every new type of biological material under investigation. A full account of apparatus and techniques and the modified theory of the determination of the dry weight will be given elsewhere.

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Experimental Cell Research, **6**, *540–542* (*1954*)

STUDIES ON THE QUANTITATIVE DISTRIBUTION OF THE DRY WEIGHT IN SQUAMOUS EPITHELIUM DURING CARCINOGENESIS

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Changes in the chemical composition of squamous epithelium during carcinogenesis have been extensively investigated (1). However, the results obtained from chemical analyses of the whole epidermis can hardly be interpreted in terms of changes in the different layers or in the individual cells. By means of microincineration, considerable demineralization has been demonstrated in precancerous and regenerative hyperplastic epidermis (6). X-ray historadiographic studies have shown variations in the distribution of the dry weight in the different layers of hyperplastic and cancerous epidermis (4). This is a preliminary report of a study of the quantitative distribution of the dry weight in squamous epithelium during carcinogenesis with the use of X-ray absorption spectrophotometry (2, 3).

Precancerous hyperplasias and epidermoid carcinomas were induced in the vaginal epithelium and in the skin of C_3H inbred mice with methylcholanthrene. Small tissue pieces, representing various stages of the changes from normal epithelium to fully developed epidermoid carcinomas, were frozen-dried with the equipment and technique described previously (5). Some tissue pieces were also fixed in 10 per cent neutral formalin for 48 hours or in Carnoy's solution for 3 hours. Frozen-dried and formalin-fixed fresh biopsies from human patients also served as material for the investigation. Each of the pathologically changed tissue pieces was embedded together with a piece of the homologous, normal, identically treated tissue in the same block of paraffin. The two tissues could thus be cut in the same section. Sections, 5–7 μ thick, were mounted on metal holders as described by Engström and Lindström (2). For details regarding the X-ray spectrophotometric technique used see Lindström (3). In this study the results were obtained either as the absolute dry

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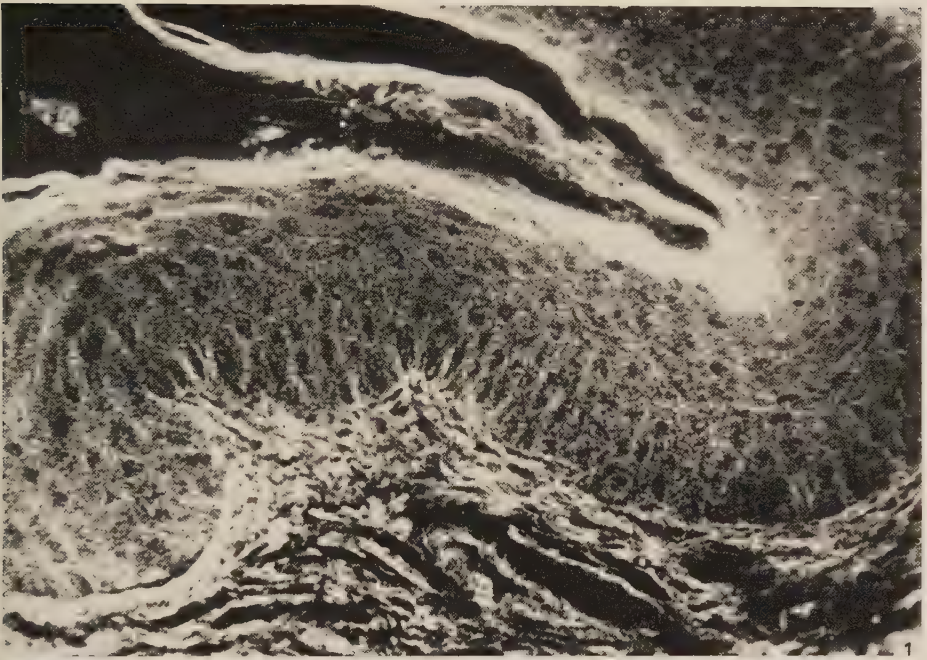
Fig. 1. Microradiogram of a 5 μ section of normal vaginal epithelium from a mouse. Secondary magnification 375 ×. Light areas correspond to structures with a high dry weight. Note the fairly homogeneous X-ray absorption in the different mucous layers and the rather high absorption in the thin keratinized layer at the surface.

Fig. 2. Microradiogram of an epidermoid carcinoma induced in the vaginal mucosa of a mouse. Secondary magnification 375 ×. A great variation in the X-ray absorption in different parts can be seen. In the basal cell layers and in the keratinized areas of the inner parts of the cancer cords the absorption is rather high corresponding to a high dry weight. In the rest of the cords the absorption is obviously low. Note the nucleoli in the different layers.



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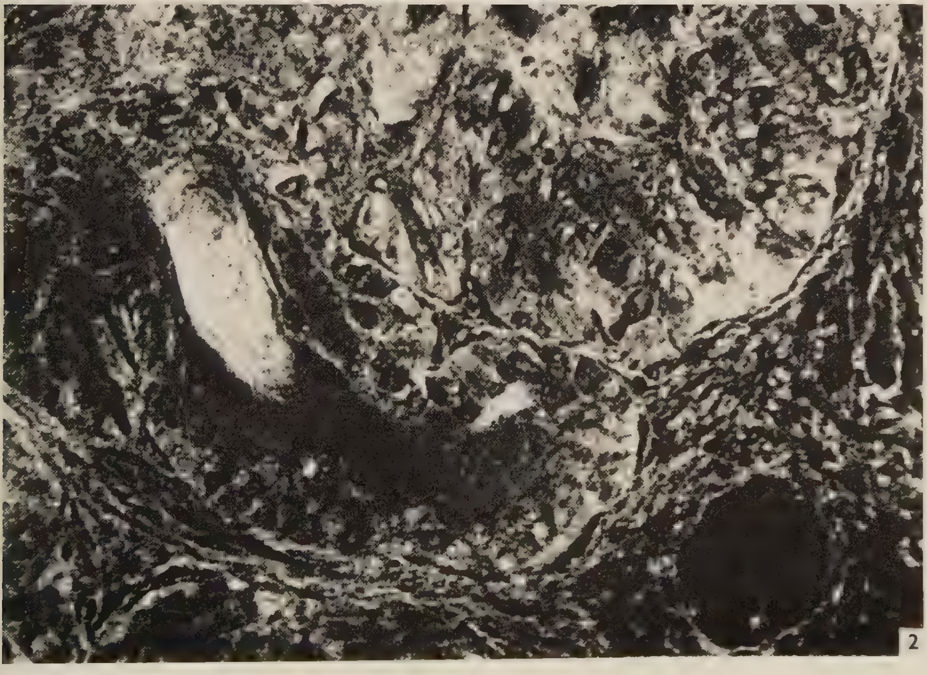

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weight per unit area or as the ratio between the mean dry weights of the two different tissues in the same section. This ratio is independent of the thickness of the section.

The error in the procedure used was determined from a series of sections of normal and hyperplastic vaginal epithelium from mouse. The standard error of a single determination of the ratio between the mean dry weights of the two types of epithelium was ±8 per cent. Local variations in the thickness within the same section, the biological variation and the random error of the method are included in this figure. The standard error of the determination of the mean dry weights per unit area was about ±5 per cent, including the error in the reference system. As the two tissues to be compared are cut in the same section, variations in the dry weight can be determined as ratios between dry weights per unit area. Hence, the determination of the thickness of the sections is not necessary.

The effects of the treatment prior to the absorption measurements have to be considered for each type of material under investigation. When different types of squamous epithelium were fixed in formalin or in Carnoy's solution, the dry weights per unit area were, in general, found to be 10–15 per cent higher than those in the corresponding frozen-dried specimens.

In the spinous layer of the hyperplastic epithelium a decrease in the dry weight of about 15 per cent was found as compared with the homologous, identically fixed, normal epithelium. This decrease in the dry weight was still more pronounced in central areas of many solid epidermoid cancer cords, but increased values of the dry weight were also frequently seen in these due to irregular keratinization (see Figs. 1 and 2). The basal layers of hyperplastic and of normal epithelium had the same dry weight per unit area. In many cancer cords, however, the dry weight was higher in the peripheral cell layer next to the connective tissue.

The investigation has shown that considerable changes in the distribution of dry weight occur in squamous epithelium during carcinogenesis. A complete account of this study will soon be published.

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A SUBMICROSCOPIC VESICULAR COMPONENT OF SCHWANN CELLS AND NERVE SATELLITE CELLS[1]

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Palade (3) has recently described a vesicular component in the endothelial cells of blood capillaries. This component was recognizable in the form of spherical or oval vesicles of globules about 650 Å in diameter. These vesicles could be seen throughout the cytoplasm of the endothelial cell, but were most abundant in the cytoplasmic region immediately adjacent to the inner and outer capillary cell membranes, where many of them were observed to open into the capillary lumen or into the pericapillary intercellular space. Palade suggested that these vesicles might bear a relation to capillary permeability. More specifically, he suggested that they might represent a system for transporting fluids across the capillary wall by a process analogous to that of pinocytosis described by Lewis (2). In Lewis's classical paper and motion picture depicting this process, cells in tissue culture were observed to take in globules of fluid from the surrounding medium, the uptake of fluid being accompanied by vigorous cytoplasmic motion at the edge of the cell, as if globules were being surrounded and engulfed by clasping folds of cytoplasm. Globules so taken in at the edge of the cell could then be observed by means of the light microscope to be transported as tiny vesicles to other portions of the cell several microns away. Since then pinocytosis has become a familiar phenomenon to observers of a wide variety of living cells in tissue culture or in transparent chambers. Palade interpreted his electron micrographs as suggesting that a similar process might be occurring in capillary endothelial cells, but involving vesicles of submicroscopic dimensions.

The purpose of this note is to confirm Palade's findings with respect to blood capillaries, to concur in his interpretation, to show that Schwann cells and satellite cells of sympathetic neurones contain a similar vesicular component and to suggest that these cells may likewise transfer materials in an analogous manner.

Sympathetic ganglia of the leopard frog (*Rana pipiens*) and of the bullfrog (*Rana catesbiana*) and the gracilis muscle of the mouse were fixed in Palade's (4) buffered osmic fixative, embedded in methacrylate and sectioned. Thin sections studied in the electron microscope revealed numerous capillaries showing the vesicles described by Palade (3), varying in diameter from 350–650 Å (mean of 26 measurements, 520 Å). These vesicles can be noted in Fig. 1 (a section from the sympathetic nerve ganglion of *Rana pipiens*), and in Fig. 4 (from the sympathetic ganglion of *Rana catesbiana*).

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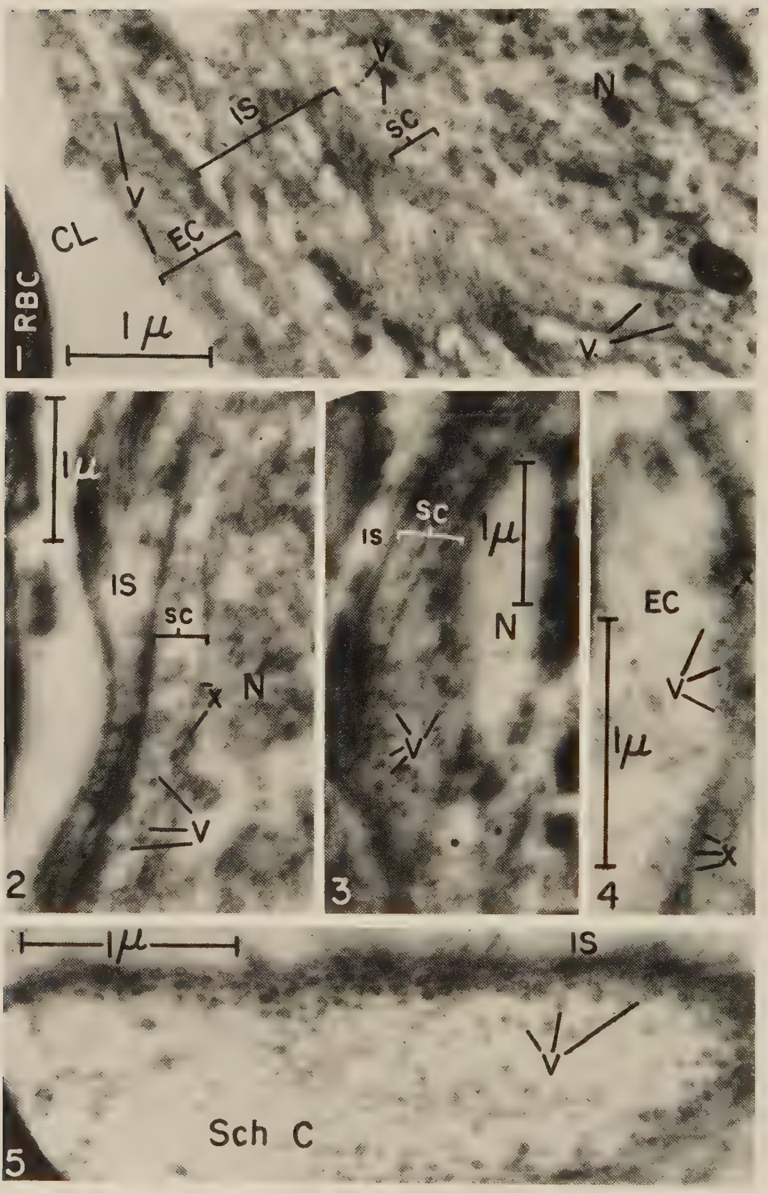
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All figures are electron micrographs of buffered osmic fixed sections of portions of sympathetic ganglia of the leopard frog (Figs. 1, 2 and 3) and bullfrog (Figs. 4 and 5).

Key: Cl — capillary lumen; EC — endothelial cell; IS — intercellular space; N — nerve cell body; RBC — red blood cell; SC — satellite cell; SchC — Schwann cell; V — vesicular component; X — sites of stomata or openings of vesicles.

The vesicles appear in greatest abundance at the capillary cell surfaces, particularly at the pericapillary surface. Many of the vesicles are oval or elongated, and some can be seen to open through tiny stomata or tubules into the pericapillary space (see X, Figs. 1 and 4).

In Fig. 1 the capillary cell just referred to borders on a connective tissue space containing collagen and other connective tissue components. This space is in turn bordered on the left by a satellite cell attendant to a sympathetic ganglion cell, a portion of which occupies the upper left corner of the figure. Vesicles similar to those in the capillary are demonstrated within the cytoplasm of the satellite cell. Vesicles of this nature are likewise to be seen in the portions of satellite cells from the leopard frog shown in Figs. 2 and 3. Here some of the vesicles can be noted piercing the satellite cell wall by small tubules or stamata (at X), and similar vesicles can be seen within the neuronal cytoplasm in Fig. 2. Fig. 5 shows a tangential section through a portion of a Schwann cell from the sympathetic nerve of the bullfrog. A striking array of globules or vesicles is shown, most abundantly at the outer cell surface of the Schwann cell. Measurements of the diameters of 23 vesicles in satellite cells averaged 680 Å, and of 30 vesicles in Schwann cells yielded a mean of 510 Å.

It is possible that these vesicles were encountered in Schwann cells by Hess and Lansing (1), who describe "circles" 300 500 Å in diameter in the "neurilemma as it inflects at the node". They suggest that these "holes" may be "pores or extensions ... of the neurilemma or membrane of the cell of Schwann", and cite indications that such structure may exist elsewhere in the Schwann cells. However, their micrographs as reproduced do not show resolution or contrast sufficient to justify a certain conclusion regarding the identity of these "circles".

We are receptive to Palade's suggestion that the capillary vesicles may represent fluid globules in process of transport across the capillary cell, arguing that since uptake and transport by cells of fluid in vesicles of microscopic size is such a familiar and widespread phenomenon, it is altogether reasonable to postulate an ability of cells to move fluid in a similar manner in vacuoles of submicroscopic size. The very close resemblance of the vesicles in the Schwann cells to those in capillaries implies a similar function, and suggests that the Schwann cells and satellite cells may mediate exchange of materials between nerve cell and intercellular space in part by pumping material in tiny vesicles from one surface of the cell to the other, perhaps working the intravesicular contents over in passage, selectively adding to or removing substances along the way. These observations imply that the permeability properties of cellular membranes may be very complex, and are not likely to be described adequately by considerations based on assumptions that such membranes function mainly as simple filters characterized by pores of certain sizes, as postulated by Pappenheimer (5).

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ELECTRONMICROGRAMS OF LARVAL AMPHIBIAN EPIDERMIS[1]

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During experimental studies on the fine structure, formation and repair of the basement lamella of amphibian skin, the following features of the adjoining epidermis cells were observed under the electronmicroscope. Objects: Premetamorphic urodeles (*Amblystoma opacum*) and anurans (*Rana pipiens*). Fixation: Veronal-buffered (7.4) osmic acid. Ultrathin sections (cca. 0.1 μ) after embedding in a mixture of methacrylates.

The basement lamella (b.m.) consists of 20 (± 2) layers of straight collagen fibers stacked crosswise (2). It is set off against the epidermis cell (ep.c.) by a continuous sharp interface (Figs. 1, 2, 6). Proceeding into the interior of the ep.c., one notes first a rather hyaline membrane (m; Fig. 1, 3, 6), containing a single layer of denser granules (g; Figs. 3, 6); next, a thin layer of ectoplasm spanned by peculiar "bobbin"-shaped bodies (b; Fig. 1, 3, 4, 6); and finally, the endoplasm (e) which has a cotton-like, furry, appearance.

"Bobbins" (more sharply outlined in urodeles than in anurans) consist of a heavily electron-absorbing base, a similar top, and a less dense connecting piece; the sagging contour of the latter may be fixation artifact. Their density indicates metal content (phosphorus?), but their role is obscure; since they occur exclusively at the border facing the b.m. they might be related to fibrogenesis or to the mechanism of attachment (Fig. 6).

The endoplasm appears to contain a continuous feltwork of finest filaments (Fig. 1, 2). On closer study, it appears, however, that the "filaments" are actually the contours of densely packed columns or canals of about 200 Å diameter, as schematized in the diagram, Fig. 5; whether anastomosing or not, could not be resolved. This interpretation seems supported by preparations from which the embedding medium had been removed (by toluene), leaving a coarse distorted fabric of beaded cytoplasmic "filaments" of likewise about 200 Å diameter (Fig. 4) a negative, as

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[1] Supported by grants from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

Fig. 1. Basal part of epidermal cell of larval urodele. × 42,000.

Fig. 2. Basal part of epidermal cell (ep.c.) with adjoining basement lamella (b.m.) and underlying mesenchyme cell (m.c.) (n, nucleus). Anuran larva. × 20,000.

Fig. 3. Diagram of border region of epidermal cell (ep.c., stippled) and basement lamella (b.m., striped; with collagen fibrils).

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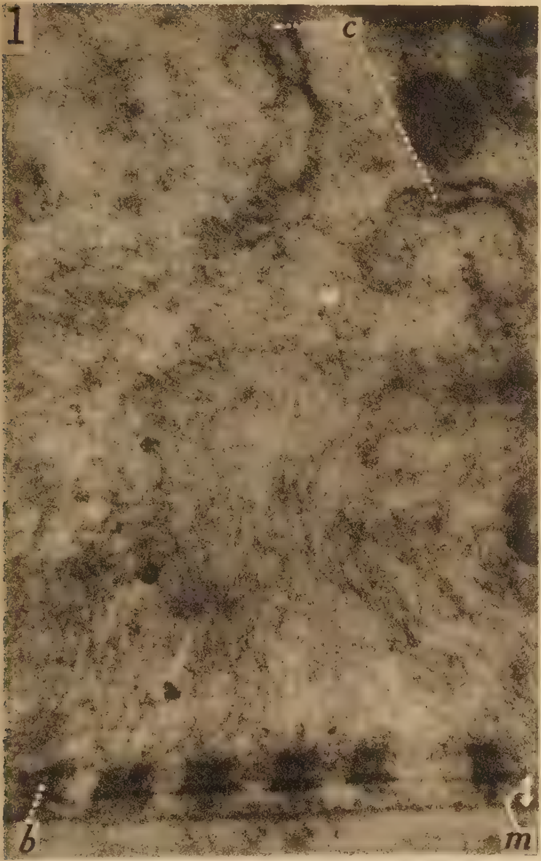

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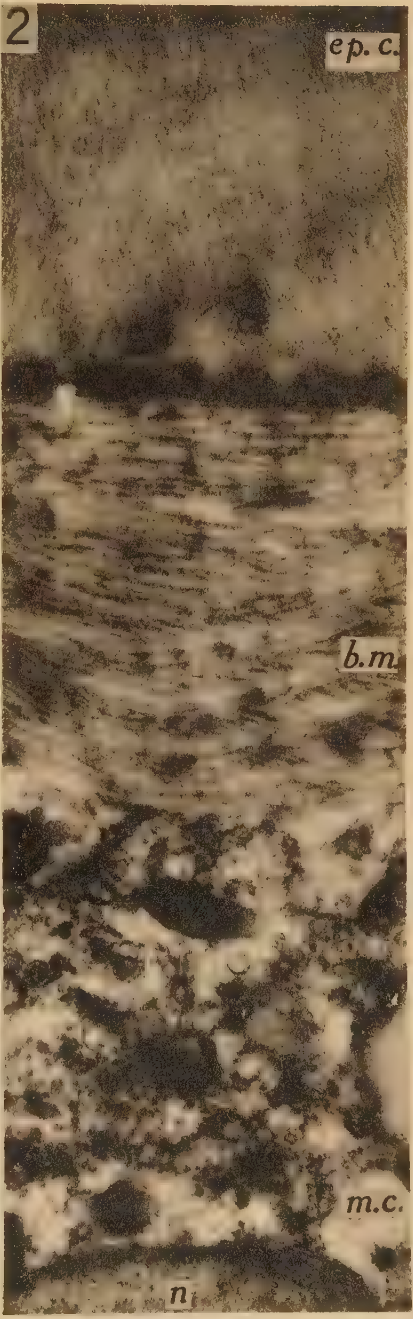

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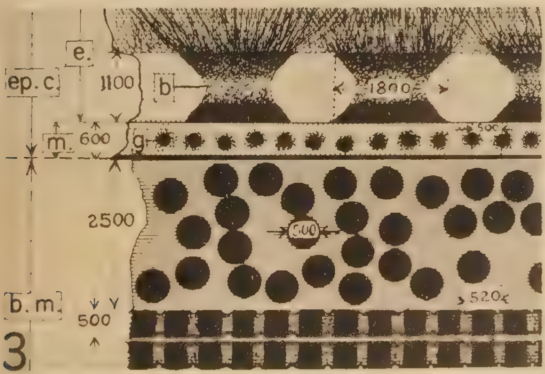
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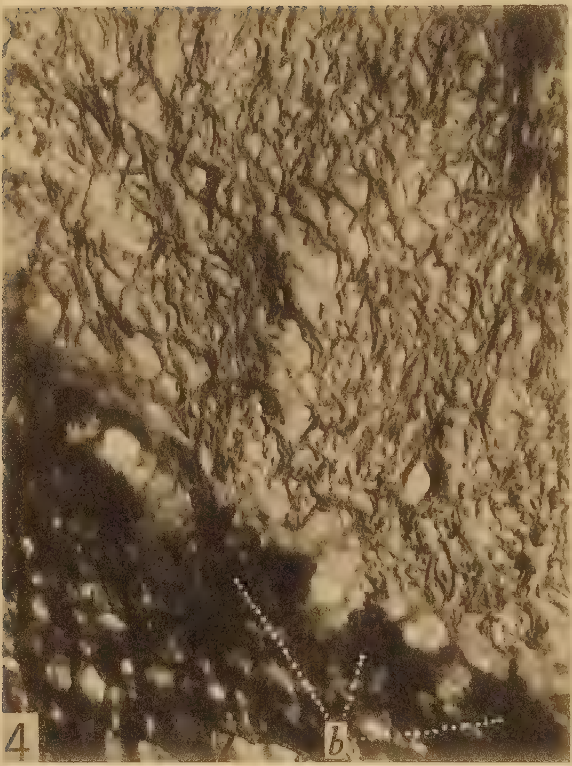

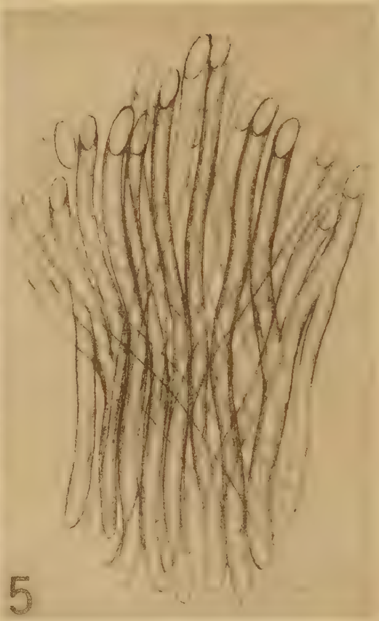

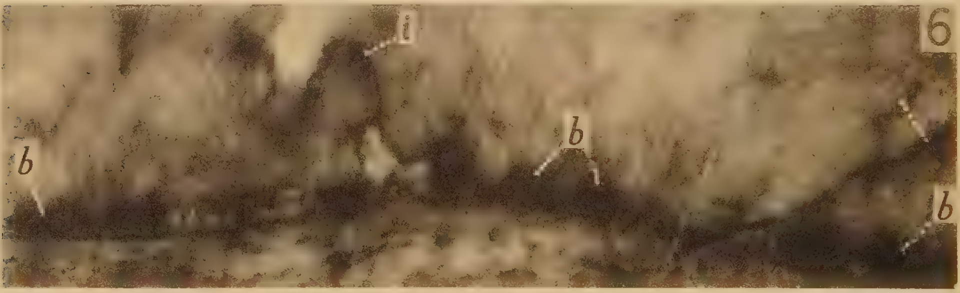

Fig. 4. Basal part of epidermal cell and adjoining basement lamella (anuran larva) after toluene removal of methacrylate and chromium shadowing. ×33,000. Note coarse cytoplasmic filaments; blurred "bobbins" (b) and orthogonal fabric of collagen fibers in b.m.

Fig. 5. Diagrammatic sketch of presumed arrangement of the cytoplasmic filaments or canaliculi (cca. 200 Å wide) of the ep.c.

Fig. 6. Basal border of three anuran epidermal cells (i, cell boundaries), showing confinement of "bobbins" (b) to surface of basement lamella. ×27,400.

it were, of the former picture. We also noted some larger canals and lacunae (cca. 800–1000 Å) (c; Fig. 1), perhaps corresponding to Porter's (1) endoplasmic reticulum, but with no obvious relation to the finer texture described above.

In contrast to the ep.c., the mesenchyme cell (m.c.) possesses no double-walled membrane, its cytoplasm is granular, without trace of the "furry" appearance of the ep.c. (Fig. 2), and it contains more mitochondria.

The dimensions given in Fig. 3 are in Ångström units and represent approximate averages.

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Experimental Cell Research, 6, 550–553 (1954)

CELL CRUSHING: A TECHNIQUE FOR GREATLY REDUCING ERRORS IN MICROSPECTROMETRY

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Microspectrometry is one of the most important methods for measuring the total amount and distribution of substances in individual cells (e.g. 2, 3, 8). While in biochemistry the reliability of using light absorption on a macro-scale as a measure of the quantity of absorbing substance in solutions is well established, in microspectrometry of cells, owing to the nature of the geometry and absorption of the object, the measurements are of uncertain validity. The purpose of this note is to describe a very simple method of greatly reducing the errors in microspectrometry due to the physical nature of the object. The method renders the solid mass of absorbing material commonly used in microspectroscopy almost as amenable to accurate measurement as a solution in a parallel-sided absorption cell. As a result, many of the doubts which have pervaded the subject of microspectrometry for a number of years should disappear.

Errors in microspectrometry may be summarised roughly as follows. Unless the optical density of the specimen is everywhere low (~ 0.1), or its optical density is uniform and its shape is simple (and this is generally not so at all), there is no simple way of determining the total amount of absorbing substance in a cell or one of its parts. The main errors arise from 1) parts of the object being out of focus; 2) glare; 3) image deformation due to diffraction from parts of the object which are small; 4) light scatter. The magnitude of these errors is often uncertain. When the object lies approximately in the plane of the slide, and thus may be in focus over the whole field, the total amount of absorbing substance may be obtained by integrating the optical density over all parts of the object. Unless special scanning apparatus is used (4) such integration is laborious and may be inaccurate.

Various techniques (6, 7, 9) of some degree of complexity and of doubtful reliability have been devised to eliminate some of the above errors. A very simple method, however, is as follows: The cell is compressed and flattened so that the whole absorbing region is converted into a thin sheet of low optical density (~ 0.1) lying in the plane of the slide. The total light energy absorbed by the region when uniformly illuminated is approximately proportional to the total quantity of absorbing substance. Provided that the optical density of the crushed cell can be made sufficiently low, no measurement of irregular areas or integration of optical density is required, errors due to glare and diffraction become negligible, precise focus is not necessary, and aberrations in the microscope objective have little influence on the measurements. Light-scatter in fixed cells is greatly reduced after compression because the solid parts of the cell are pressed together and the fluids squeezed out.

Suitable flattening and compression of cells may conveniently be achieved by a technique (1) developed for crushing mineral particles while being viewed in the microscope. The specimen is mounted on the underside of the cover slip and covered by a sheet of cellophane, ~25 μ thick. An immersion condenser is used with a special top lens having a convex spherical surface of 0.5 mm radius at the focus (Fig. 1). The cover slip is held on the microscope stage by means of a metal plate, and when the condenser is centred and racked upwards the specimen is compressed by the top lens of the condenser. Slight pressure causes flattening of cells similar to that achieved by standard cytological techniques (Fig. 2b). Greater pressure causes individual chromosomes and other small bodies to be themselves flattened so that their optical density is reduced two or three times (Fig. 2c). Measurement of the total quantity of absorbing substance Q in the cell is then extremely simple and is made as follows. The field stop diaphragm is adjusted (diameter D) to contain the object, and a measurement of total light transmission I_t is made with a photocell. The object is then moved out of the field and the light I_0 passing through a clear field is measured. The difference between the readings is roughly proportional to the amount of absorbing substance in the object. This method is similar to that used by Pollister and Ris (8), but which was later largely abandoned owing to the unsuitable nature of most absorbing bodies in cells.

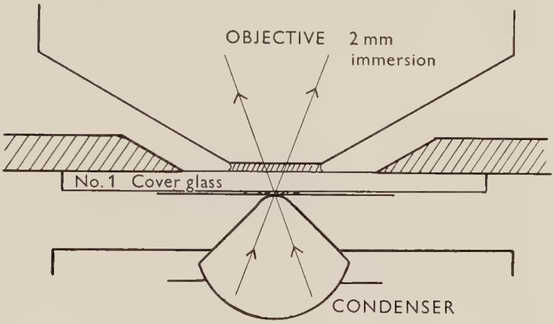

Fig. 1. Diagram showing top lens of crushing condenser, object, cover glass, metal plate holding cover glass, and microscope objective.

The relation

$$Q \propto (I_0 - I_t) \qquad 1)$$

is not exact; in general

$$Q \propto \log_{10} I_0/I_t \cdot D^2 \qquad 2)$$

is more nearly exact.

If the optical densities of parts of the object, although small, are greater than 0.1 it may be worthwhile to make a small correction; e.g. if the object is of optical density 0.2 and occupies about half the area of the field, the remainder being clear, Q obtained from 2) should be increased by 11 per cent (see formula given by Glick, Engström and Malmström (5)). It may be noted that often relative values only of Q are required and then the errors are less. For instance, if the optical density of an object which occupies half the area of the field is 0.1 and in another case 0.2 and the values of Q derived by means of 2) are Q_1 and Q_2 respectively, the ratio Q_1/Q_2 is in error by only 6 per cent if the correction is not made.

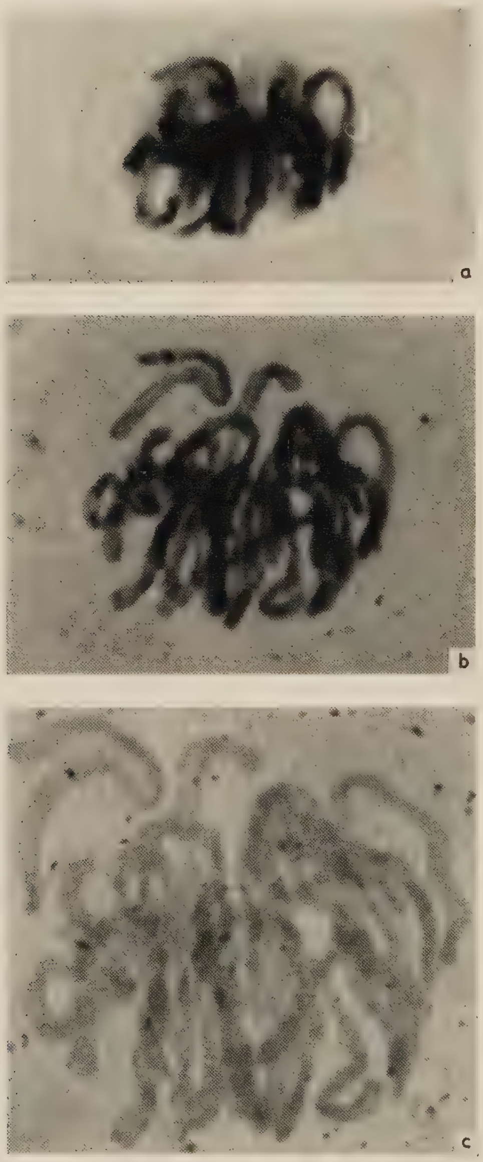

Fig. 2. Cell at metaphase, in squash preparation of onion root tip; fixed acetic-alcohol. Feulgen stained and mounted in glycerol; Mag. ×1800, 546 mμ. Objective numerical aperture 1.2.
(a) Cell before crushing.
(b) Cell after slight crushing.
(c) After further crushing.
Most regions have an optical density ~0.15; some small parts are 0.3.

We consider that the cell crushing technique helps greatly to place microspectrometric measurements on a firm basis and should enable many biologists to obtain reliable data by using simple apparatus. The cell-crushing technique, of course, in no way reduces the often very real uncertainties in microspectrometry caused by lack of specificity of absorption curves, lack of stoichiometry in staining reactions, etc. A constant light source and a non-fluctuating light measuring instrument is required to measure the low optical densities of crushed cells. So far we have tested the method only in visible light on stained cells; but clearly it should apply in the ultraviolet. The work is continuing.

It may not always be feasible to crush a cell sufficiently so that the simple procedure of measurement we describe is accurate. The extent to which it may be preferable or necessary to use scanning integration methods (4) can be judged as research proceeds. Apart from applications to absorption measurement, cell crushing also appears to be a useful technique for micromanipulating cells and for revealing morphological detail.

We wish to thank Professor J. T. Randall for encouragement and Mr. Setterington and Messrs. R. and J. Beck Limited for designing and constructing the crushing condenser.

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Experimental Cell Research, **6**, *554–556 (1954)*

IRREGULARITIES OF CHROMOSOME DIVISIONS IN *TRADESCANTIA* GROWN ON LOW SULFATE[1]

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Sulfur is one of the major elements required for growth. In mineral nutrition experiments little attention has been given to studies of the nucleus. The experiments here deal with abnormalities of nuclear division from plants grown on low sulfate, which may further delimit the role of sulfur in the celll.

Methods for a sulfate deficiency in nutrient solution were followed (2) except that some magnesium sulfate was added to insure flowering and limited growth. Two low sulfate levels, 8.0 ppm and 4.0 ppm, were employed along with normal Hoagland's solution controls (solution 1 with 192 ppm sulfate) using 10 plants per treatment. The plants of *Tradescantia paludosa* (clone 5 of Sax) were grown for approximately 7 months. Inflorescences were collected for examination of meiotic and microspore divisions 4 to 7 months after the start of the cultures. Root tips were collected in the last month only. Anther material was stained with propionic carmine, while in root tips the Feulgen technique was used.

Material from the culture containing 4.0 ppm sulfate was the first to show abnormal cytological effects. The 8.0 ppm sulfate culture also showed the same symptoms but at a later date. Both mitotic and meiotic chromosomes from control plants divided normally and showed no abnormalities. However, in low sulfate treatments chromosome division was definitely abnormal. As seen in Fig. 1 a, the six bivalents did not separate at first anaphase of meiosis and each bivalent has formed a micronucleus. Less severely affected cells (second anaphase) exhibited unequal chromosome distribution and chromosome laggards, as seen in Fig. 1 b. Tripolar spindles were often observed. For the most part the low sulfate material appeared as if spindle function was severely impaired. This phenomenon is usually termed C-mitosis (C-meiosis here). Drastically influenced meiotic cells, seen in Fig. 1 c, show nuclei of various sizes, the smallest being unseparated bivalents. Microspores with the diploid number of chromosomes were found. Also in some cases extra large pollen grains with large nuclei were observed, which perhaps indicates a tetraploid condition. This too can be taken as evidence for impairment of spindle function.

Somatic chromosomes were similarly influenced but less severely. In root tips micronuclei were present, as shown in Fig. 1 d. Generally the micronuclei were uniform in size and equivalent to whole chromosomes. In a few cases one micronucleus was smaller than the others, as in Fig. 1 d, which would indicate that chromosome

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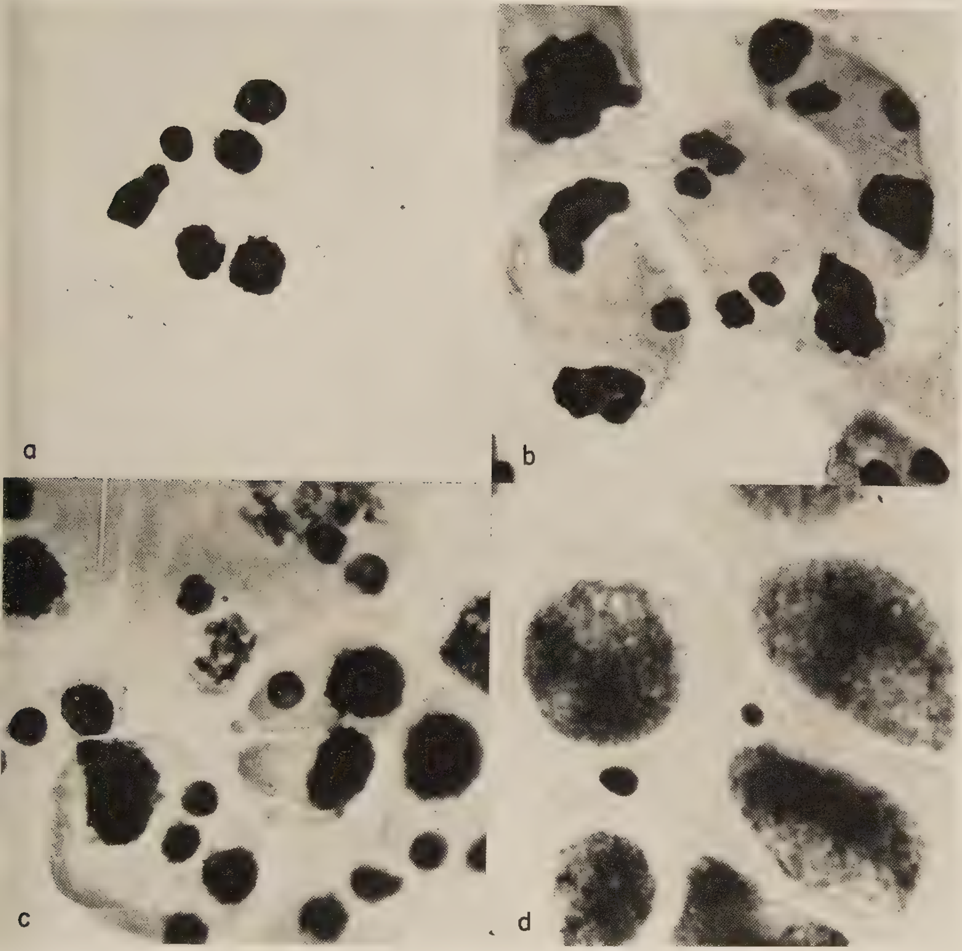
[1] Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.



ig. 1. The cytological effects produced by a low sulfate nutrition in *Tradescantia*.
) Six bivalents at first metaphase of meiosis have formed micronuclei. Magnification = 920 ×.) In second anaphase of meiosis lagging and unequal distribution of chromosomes is seen. agnification – 1070 ×. c) Meiotic nuclei are of unequal sizes. The smallest are unseparated ivalents. Magnification = 665 ×. d) Two micronuclei of different sizes are present in somatic ells of root tips. Magnification = 665 ×.

reakage had occurred. The progeny of low sulfate plants are being examined to test hromosome mutations produced.

In addition to nondisjunction and irregular distribution of chromosomes, meiosis nd microspore mitoses are slowed down or stopped in cells lacking sulfate. This vidence was obtained by comparing the length of the flower pedicel with the stage f its microspores. Sections from control pedicels (both young and mature) showed that some cell division was going on, but that cell elongation was the main component of growth. The pedicel length serves mainly as in indicator of time. In control buds when microspores were at metaphase of the first mitotic division, the pedicel length varied from about 0.1 to 2.5 mm in length. With both levels of low sulfate, buds bearing microspores in first mitotic divisions were borne on pedicels that were 5.0 to 10.0 mm long. In some cases open flowers had microsporocytes in the first division, while controls would be dehiscing mature pollen. In other mineral deficiencies (magnesium or iron) at microspore division the pedicel lengths were within the range of controls.

Two types of nuclear disturbances induced by chemical means have been described by Levan (3). One group of compounds produces mitotic inhibition and/or C-mitosis as does the sulfate deficiency. The second group of chemicals (mutagens) produces chromosome breaks. Chromosome breaks were induced by a magnesium deficiency in *Tradescantia* (7).

In reference to heritable changes, a sulfate deficiency increased the mutation rates in snapdragons (8).

It is not unexpected that a sulfate deficiency should produce mitotic inhibition and nuclear disturbances. Sulfhydryl substances have long been thought to be involved with nuclear division (7). It is probable that sulfhydryl groups are present in the spindle (4). In *Juglans* deficient for zinc mitosis was arrested. When cysteine was added the mitotic activity increased (6). Using *Vicia faba* roots cultured in S^{35}, the sulfur was found associated with chromosome material other than heterochromatin (5).

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Experimental Cell Research, **6**, *557–559* (*1954*)

MEIOTIC-LIKE DIVISIONS IN NORMAL MYELOCYTES

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During a study of leukopoiesis in an inbred strain of the Japanese albino rat several instances of meiotic-like division figures were noted. Their occurrence seems to be sufficiently interesting a biological occurrence to warrant the publication of a short note. Somatic reduction and segregation have been reported previously in several species of plants and animals (1, 3, 4, 5, 6) and in tumor cells (2).

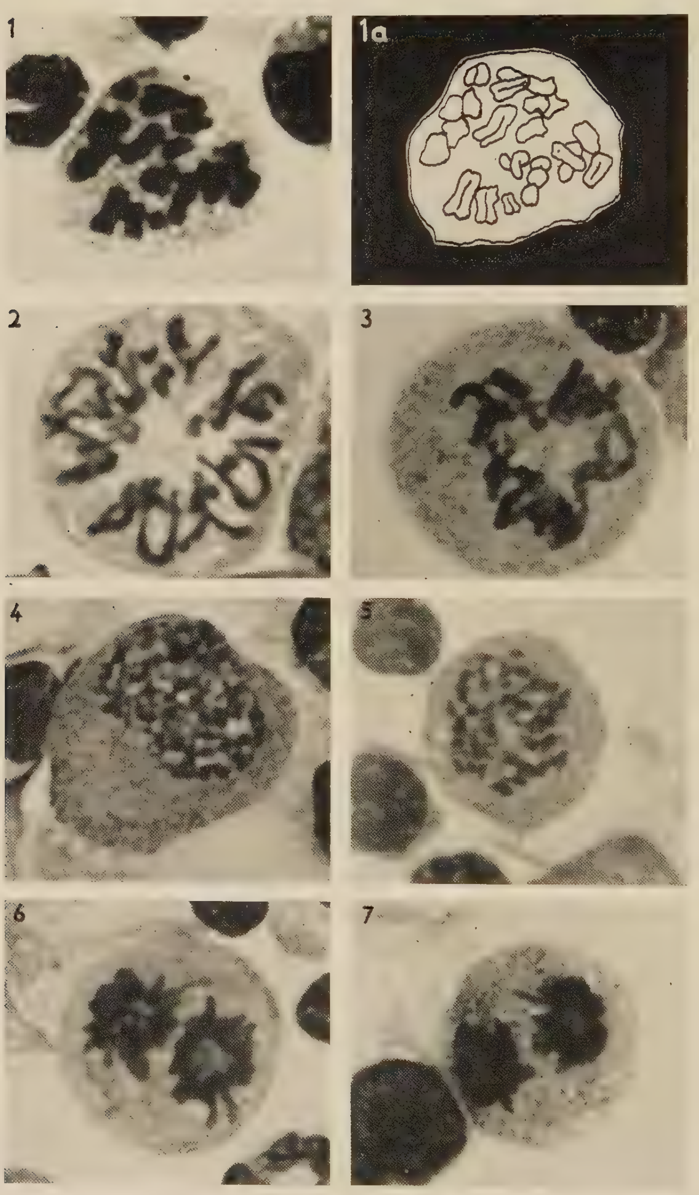
Samples of fresh, normal bone marrow were obtained from the excised left femur of normal rats and suspensions of marrow cells were obtained by rinsing bits of the marrow in horse serum. This technique minimized mechanical injury to the cells and also resulted in an even distribution of cells in the films prepared for microscopic examination a better film than is obtained by touch preparation techniques. The preparations were fixed for 10 seconds with one per cent chromic acid solution followed by methanol for three minutes. Giemsa's stain was employed because it permitted classification of the various types of myeloid cells on the basis of staining characteristics of cytoplasm and granules.

Fig. 1 illustrates the metaphase stage of meiotic-like division of a promyelocyte. A comparison of this with Fig. 2, which pictures the usual mitotic metaphase observed in the rat myelocyte, serves to illustrate the meiotic-like aspect of this division figure.

Among the myelocytes there exist cells which are apparently haploid in constitution. These cells are smaller with correspondingly smaller nuclei than the other myelocytes. Several division figures have been found among these smaller cells which permit an accurate count of the chromosome number to be made. In such cases the haploid number of 21 has been found.

Fig. 4 shows a prophase figure of what is believed to be a diploid cell and Fig. 5 illustrates a prophase of what is believed to be a haploid cell. Similarly the metaphases of Figs. 2 and 3 may be compared. And lastly the anaphase pictures of Figs. 6 and 7 illustrate a diploid and a haploid myelocyte, respectively. All photographs were made at the same magnification.

The significance of these haploid cells is not known but whatever their function, it is suggested that the meiotic-like figures observed during this study may be the process through which a precise quantitative and qualitative distribution of chromatin material is made to these haploid cells.



Photomicrographs of myelocytic cells: Fig. 1, promyelocyte; Figs. 2–7, semimature myelocytes. Fixed and stained as described in text. Photographed at 1350 magnification.
Fig. 1—meiotic-like metaphase. Fig. 1a—line drawing of Fig. 1 illustrating 21 bivalents. Fig. 2—diploid metaphase. Fig. 3—haploid metaphase. Fig. 4—prophase diploid cell. Fig. 5—prophase, haploid cell. Fig. 6—anaphase, diploid cell. Fig. 7—anaphase, haploid cell.

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MORPHOLOGISCHE UND CYTOCHEMISCHE BEOBACHTUNGEN AN TABAKMOSAIKVIRUS-INFIZIERTEN PROTOPLASTEN VON *NICOTIANA TABACUM*

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Verfolgt man die intraplasmatischen Veränderungen in individuell mit Tabakmosaikvirus (TMV) infizierten Haarzellen von *Nicotiana tabacum* (13) oder in Zellen von Blättern, die nach mechanischer Inokulierung mit TMV unter weitgehend konstanten Bedingungen gehalten wurden (25° C, 8000 Lux, komplette Nährlösung), so lassen sich leicht vier Stadien verschiedener Infektivitätsqualitäten abgrenzen (N. glutinosa-Test, 7). 1. Während der Periode verstärkter Zellaktivität (Cyclosis) bis zu beginnender X-Körper-Bildung sind keine infektiven Substanzen zu isolieren. 2. Mit dem Auftreten von einem oder mehreren amorphen X-Körpern erreicht die Infektivität der Isolate ihren Höhepunkt, um dann 3. nach Abbau der X-bodies und mit dem Erscheinen intraplasmatischer Kristalle progressiv abzusinken. 4. Im Spätstadium der Infektion, wenn die Kristalle aufgelöst oder von kristallinen Fibrillen ersetzt werden, ist nur noch schwache Infektivität nachweisbar. Der durch diese bekannten Stadien grob markierte Gesamtverlauf der Zellinfektion zeigt nun im einzelnen an lebendem und gefriergetrocknetem (9) Material auffallende morphologische Veränderungen, die zum Teil auch von cytochemisch fassbaren (3, 4) substantiellen Konzentrationsschwankungen im Protoplasten begleitet sind. Während der ekliptischen Periode der Virusvermehrung wandeln Zellkern und Nukleolus in bestimmtem Rhythmus Grösse und Gestalt. Frühestens 4, spätestens 20 h nach Inokulierung zeigt sich der Zellkern von einer zunächst scharf umgrenzten und später diffuser erscheinenden Zone U. V.-absorbierender Substanz (2600-2900 Å) umgeben. Mit dem Auftreten zahlreicher, im gleichen Wellenbereich stark absorbierender Partikel, die vom strömenden Plasma mitgeführt werden, verschwindet dieser Absorptionsmantel wieder. In diesem, für die frühe Virusinfektion typischen Infektionsstadium, das oft tagelang andauern kann, ist eine auffallende Reduktion des Kernvolumens bemerkbar, die sich auch U. V.-spektrographisch in der Abnahme der Gesamtabsorption des Zellkerns ausdrückt. Mit beginnender X-Körper-Bildung nimmt das Kernvolumen wieder langsam zu und bleibt während der X-body-Periode relativ konstant, aber immer unter den Proportionen vergleichbarer gesunder Kerne. Mit dem Auftreten von Kristallen innerhalb der zerfallenden X-Körper oder in bestimmten Zonen des Cytoplasmas ändern sich die Kernverhältnisse von neuem. Kern und Nukleolus können bis zum Vierfachen ihres ursprünglichen Volumens anschwellen. Die U. V.-Extinctionswerte zeigen auch hier eine Massenzunahme an. Im Spätstadium der Infektion werden die Kristalle wieder aufgelöst, die Plasmastränge verlaufen dann in eigenartig geraden und starren Bahnen. Meist fallen in ihnen flexible kristalline

Fibrillen aus, die sich zu Strängen vereinigen können und mit der Zeit wieder verschwinden. Die Phänomene der Kristallbildung und des Kristallabbaues können sich in der gleichen Zelle mehrfach wiederholen.

Bei der Beobachtung der Kristallbildung in lebenden Zellen (Phasenkontrast 1500 ×) wurde folgender morphologische Befund gemacht: In örtlich begrenzten, fein vernetzten Zonen des strömenden Plasmas entstehen dichtere bläschenförmige Gebilde von wenigen μ $\varnothing$. Diese treten fast stets in spiegelbildsymmetrischen und zunächst zusammenhängenden Zwillingsformen auf. Durch Anlagerung weiterer Schichten vergrössern sie sich zusehends zu rundlichen Kristalloiden. Der Kristallisationsprozess schreitet von aussen nach innen fort. Schliesslich entstehen regelmässige hexagonale Platten und schollenförmige unregelmässige Prismen. Auffallend ist die anomale Ansammlung fadenförmiger Mitochondrien in diesen Plasmabezirken. Die Beobachtung solcher Doppelformen liefert vielleicht einen Beitrag zu den Vorstellungen über die Anlagerung von Substratmolekülen im Kraftfeld eines Matrizensystems bei der Bildung von Proteinkristallen (5, 8, 10).

In Tabelle I sind die mittleren Extinctionswerte (2650 und 2800 Å) von mindestens je 10 vergleichbaren Zellen der beschriebenen Stadien nach Korrektur für Lichtstreuung und unspezifische Absorption in Beziehung zur relativen Infektivität entsprechender Gewebeteile gesetzt.

Auf Grund der beschriebenen wechselnden Infektivitätsverhältnisse der Zellen im Laufe der Viruserkrankung erschien es wichtig, die Virussubstanzen der einzelnen Stadien durch differentiale Ultrazentrifugierung zu isolieren. Unter konstanten Bedingungen gehaltene, möglichst gleichmässig infizierte Blattgewebe wurden zum gewünschten Zeitpunkt geerntet, extrahiert (12), die Extrakte zentrifugiert und elektronenoptisch untersucht. Bisherige Ergebnisse: Es gelang auch bei Variierung der Extraktionsmethoden und Zentrifugierungszeiten nicht, eine anomale makromolekulare Proteinkomponente von Geweben, die bis zu 20 h inokuliert waren, zu isolieren. Das Stadium beginnender X-body-Bildung ergab geringe Mengen anomaler

TABELLE I

	Kontrollen gesund		Frühstadium bis 20 h nach Inocul.		Beginn der x-body-Bildg.		X-body-Stadium		Kristall-Stadium		Fibrillen-Stadium		
	Nukl.	Plasm.	Nukl.	Plasm.	Nukl.	Plasm.	Nukl.	X-body	Nukl.	Krist.	Nukl.	Krist.	Fibr.
E 2650	.278	.02	.194	.133	.13	.136	.222	.305	.432	.292	.232	.135	.063
E 2800	.161	.032	.175	.092	.112	.105	.208	.237	.350	.273	.128	.149	.058
Anzahl Lokall. *N. glut.*	000		000		00 +		+ + + +		0 + +		00 +		

000 = keine Läsionen auf *Nicotiana glutinosa.*
00 + = nicht über 5 Läsionen auf je 10 Blatthälften von *N. glut.*
0 + + = nicht über 30 » » » 10 » » »
+ + + + = über 100 » » » 10 » » »

Nukleoproteide, die elektronenmikroskopisch als Stäbchen von vorwiegend normaler Länge identifiziert wurden. X-Körper-haltige Gewebe enthielten grosse Mengen hochinfektiver Virussubstanz, die im frischen Zustand vorwiegend normale Partikellängen zeigte. Sedimente von Material im Kristallstadium ergaben bedeutende Zunahme an Virussubstanz im Verhältnis zum Frischgewicht der Ausgangsblätter. Die Präparate enthielten aber einen auffallend hohen Anteil von Partikeln kurzer Längen (etwa 1/3). Die Anzahl kurzer Teilchen nahm in Präparaten aus länger infiziertem Ausgangsmaterial noch zu. Eine fast isodiametrische Fraktion liess sich durch differentiales Ultrazentrifugieren abtrennen. Bei pH 3,8 aggregierten diese Partikel zu den gewöhnlichen Taktoiden (11). Präliminäre Bestimmung des RNS-Gehaltes dieser Fraktion nach üblicher Hydrolysierung ergab im Beckman-Spektrometer in der 2600 Å-Bande nur ein unbedeutendes Maximum im Vergleich zu dem der „X-body-Fraktion".

Wenn die hier gefundenen nukleinsäurearmen Komponenten mit den von anderen Autoren (6, 12) beschriebenen RNS-freien Antigenen identisch sind, können sie keine frühen Aufbaustufen des TMV darstellen. Sie scheinen vielmehr in späten Stadien der TMV-Erkrankung gebildet zu werden und mögen eine Voraussetzung für die demonstrierbare Abnahme der Infektivität bei gleichzeitiger Zunahme von isolierbarer Virussubstanz sein. Die Bedeutung der Nukleinsäuren für die biologische Aktivität des Tabakmosaikvirus wird erneut unterstrichen. Andererseits lassen diese Beobachtungen darauf schliessen, dass die Synthese anomaler Proteine auch dann weiterzugehen scheint, wenn die komplementäre Rolle der RNS im proklamierten Matrizensystem der Virusreduplikation (2) nicht mehr gesichert erscheint.

Ich bin Herrn Professor Dr. T. Caspersson und der Deutschen Forschungsgemeinschaft für die Ermöglichung dieser Arbeit zu grossem Dank verpflichtet.

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AMINO ACID METABOLISM IN DEVELOPING SEA URCHIN EMBRYOS[1]

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The study of quantitative developmental changes in amino acid fractions, which has been carried out on the sea urchin *Strongylocentrotus purpuratus* (5), has been extended to *Paracentrotus lividus*. The material was collected at the Zoological Station in Naples in 1953, and the amino acid fractions were assayed microbiologically using essentially the same techniques already described (5). However, because a fractionation of the proteins was desired, a new extraction procedure has been developed. The lyophilized embryos are homogenized and extracted thrice in ice-cold 0.1N KCl-0.01N HAc. This gives a sharp fractionation of the proteins. The 0.01N HAc plays an important role in this extraction, for in its presence only 24 per cent of the proteins of the unfertilized egg will dissolve. The KCl-HAc insoluble proteins are hydrolyzed in 6N HCl at 120°C for 10 hours. The KCl-HAc extracts are lyophilized and repeatedly extracted in cold 5 per cent 0.5N HCl in acetone. This solvent extracts all dialyzable substances but the proteins are insoluble. The protein residues remaining after this extraction are hydrolyzed under the same conditions as mentioned above. The acetone is removed from the extracts by evaporation under an air-jet and the 0.5N HCl by repeated lyophilization. The final residues contain the free amino acids, peptides and other dialyzable components of the original KCl-HAc extracts. These residues are taken up in distilled water and an aliquot of this extract is hydrolyzed in 4N HCl at 120°C for 8 hours (the HCl was removed from all hydrolysates by repeated vacuum desiccation over NaOH pellets). Assays of the latter aliquot give the total non-protein amino acids while those of the unhydrolyzed extracts give the free amino acids. The difference between these values is taken to represent peptide amino acids.

Of the 13 amino acids thus far assayed, all but glycine, which is the principal osmotic component and is not considered here, show essentially the same changes with development, although minor variations do occur. The results of assays of 22 stages of embryonic development and 4 stages of aged unfertilized eggs are shown in Figs. 1 and 2. The cyclic changes in the protein amino acids, which are reported here for the first time, are believed to represent alternate periods of yolk degradation and synthesis of new embryonic proteins. There appears to be but slight overlap of these processes during the early development of *P. lividus*, and it is this fact which renders them detectible. On the other hand, such cyclic changes were not

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[2] United States Public Health Service Postdoctoral Research Fellow of the National Cancer Institute.

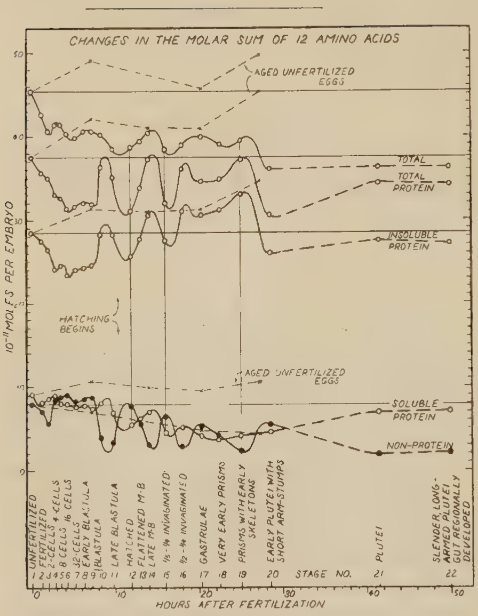

Fig. 1 (above). Changes in the molar sum of arginine, aspartic acid, cysteine, glutamic acid, histidine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine.

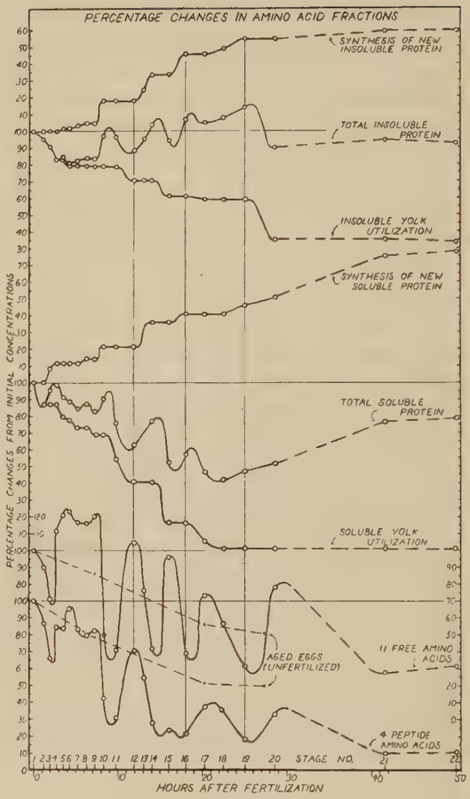

Fig. 2 (right). Average percentage changes of the amino acids from their values in the unfertilized egg (all amino acids are weighted equally). The curves for protein amino acids represent the same 12 amino acids as in Fig. 1. Cysteine is omitted from the free amino acid curve. The curve for peptide amino acids represents cysteine, histidine, phenylalanine and tyrosine.

detected in the proteins of *S. purpuratus* (5). The highly synchronous development of this batch of *P. lividus* embryos was probably promoted by fertilization in the presence of 10^{-5} M sodium periodate. This treatment greatly improves fertilization and development (6). It is probable that the metabolism of individual embryos in a large batch obtained from many females gets more and more out of step as development proceeds. If this were the case, the abrupt cyclic changes which occur would tend to cancel one another, and this probably accounts for the failure to detect the full spectrum of cyclic changes in *S. purpuratus*. It seems likely (2) that these cyclic changes correspond to the cycles of mitochondrial activity (3) and yolk granule consumption (2) observed by Gustafson and Lenicque.

Cyclic changes synchronized with but complementary to those found in the proteins occur in the non-protein amino acids, which constitute 17 per cent (excluding glycine) of the total in the unfertilized egg (somewhat similar changes were found in the non-protein amino acids of *S. purpuratus* but these were only detected before gastrulation (5)). Free amino acids account for 74 per cent of the non-protein amino acids. Asparagine and glutamine and peptide aspartic and glutamic acids make up an additional 19 per cent of this fraction. Other peptide-contained amino acids, principally cysteine, histidine, phenylalanine and tyrosine, constitute the remaining 7 per cent. The existence of complementary cycles of comparable magnitude in the non-protein amino acids indicates that in the conversion of yolk to new embryonic proteins the yolk must first be degraded to free amino acids and small peptides. There is probably very little transfer of large intact polypeptide units from yolk to new protein, for many of the amino acids are found in only relatively small amounts in the peptide fraction (the assay micro-organisms might, for example, use the valine or leucine of small peptides and thus register them as free amino acids, but they probably could not use valine or leucine bound in relatively large peptides).

Since cyclic variations occur in both protein fractions, it seems likely that at least two kinds of yolk exist. The main variations are synchronous in both fractions but there appear to be at least 3 periods (cf. the changes between stages 2 and 4, 17 and 18, and 19 and 20 in Fig. 2) when protein degradation in one fraction overlaps with the incorporation of new protein into the other.

If the cyclic changes in the protein fractions are interpreted correctly, the monotonic curves formed by summing the increasing phases should represent the synthesis of new embryonic proteins while those formed by summing the decreasing phases should represent yolk utilization. These curves are plotted in Fig. 2. They suggest that the soluble yolk is completely consumed by the time the embryos have developed into early prisms (stage 18). This would explain why there is no decrease in the KCl-HAc soluble proteins corresponding to the great drop in insoluble proteins between stages 19 and 20. The curves further indicate that the soluble proteins in the unfertilized egg consist almost entirely of yolk, while those in the late pluteus are new embryonic proteins. On the other hand, the utilization of insoluble yolk in development to the late pluteus amounts to 66 per cent of the KCl-HAc insoluble proteins in the unfertilized egg. This suggests that at least 66 per cent of the latter is yolk and at most 34 per cent is structural or other non-yolk protein.

In eggs which are allowed to stand in sea water after removal from the ovary, the amount of protein increases 21 per cent while the non-protein amino acids decrease to only 56 per cent of their initial value. These changes probably represent a continuation of the process of synthesis of yolk and other embryonic proteins which normally takes place in the ovary. They indicate that the proteins of the egg are probably synthesized by the egg itself, the ovary merely supplying the free amino acids and perhaps small peptides. The author has already shown (5) that in eggs from freshly collected *S. purpuratus* the free amino acid content is high and shows an initial drop after fertilization, whereas their content is low and increases after fertilization of eggs obtained from animals collected in season but kept for weeks under conditions which inhibit spawning. It now appears that a high content of free amino acids is characteristic of eggs in which yolk synthesis was proceeding rapidly at the time of removal from the ovary, whereas a low content is an indication that yolk synthesis had ceased, free amino acids (and small peptides) were no longer being taken up from the ovary, and the eggs were ripe and ready to be spawned. The developmental processes are sufficiently flexible so that normal development will proceed in either case. The improvement in fertilizability and development which is often obtained by letting eggs stand for hours in sea water is doubtless related to the fact that continued yolk synthesis under these conditions depletes the non-protein amino acids to the low level which appears to be characteristic of the fully ripe egg. In view of these findings, the early post-fertilization changes in non-protein amino acids (and probably other metabolites) must apparently be viewed as adjustments which bring an initially flexible system to a more rigidly defined state from which normal development can proceed.

The total molar content of 12 amino acids (Fig. 1) changes considerably during development and increases 10 per cent on aging for 27 hours. The increase amounts to 55 per cent of the non-protein amino acids in the unfertilized egg and it is 27 per cent greater than their decrease during the same period. The changes in non-protein amino acids during development do not exactly compensate those in the protein amino acids but may be of either lesser or greater magnitude. Similar discrepancies have been noted in *S. purpuratus* (5). Unless the embryos and unfertilized eggs are able to synthesize amino acids *de novo*, there would appear to be an undetected pool of amino acid skeletons or derivatives which are readily convertible to the corresponding amino acids by the sea urchin embryo, but which are not converted to amino acids by hydrolysis and cannot substitute for the amino acid requirements of the assay micro-organisms. Such a pool might represent an intermediate form through which amino acids pass in the process of being incorporated into the sea urchin proteins. In this connection Commoner *et al.* (1) have found that the amino acid residues of tobacco mosaic virus are not derived directly from the intact free amino acids of the host's pool (the bulk of the nitrogen is derived from free ammonia), while Hultin (4) has shown that the nitrogen of labeled alanine when incorporated into the sea urchin proteins is distributed throughout the amino acids.

These studies are being extended to other batches of embryos and to include other metabolites. A full report of these findings will be published later.

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Experimental Cell Research, **6**, *567–568 (1954)*

GROWTH FACTORS FOR *L. CASEI* IN DEVELOPING SEA URCHIN EMBRYOS[1]

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Gustafson *et al.* (3) have detected components in extracts of sea urchin embryos which have a growth promoting activity for *Lactobacillus casei*. They have determined the changes in this activity with development and have found them to parallel the changes in the "peptide-containing fraction" of Gustafson and Hjelte (2). This and other considerations have led them to suggest that the growth factors are peptides.

We have determined the growth factor activity in the more complete developmental series of *P. lividus* embryos used by Kavanau (6) for his studies of amino acid metabolism. The determinations were carried out on the acetone-HCl extracts used for the study of non-protein amino acids. The semi-micro assay method described by Kavanau (5) was used with the Henderson-Snell (4) medium as supplemented by Gustafson *et al.* (3). The stimulation of the early growth of *L. casei* was determined after various times of incubation. A standard curve was obtained by using serial dilutions of the test material. The average of 6 assays is plotted in the upper curve of Fig. 1. The form of this curve reproduces that of the curves obtained in all 6 assays except for minor variations. Hydrolysis of the extracts in 4N HCl at 120°C for 8 hours destroys all activity.

It is evident that the growth factors change in essentially the same way as do the free and peptide-contained amino acids (cf. Fig. 2 of (6)). The changes show the greatest similarity to those of the sum of peptide glutamic acid and glutamine (Fig. 1). The latter curve is obtained by subtracting the values for free glutamic acid from the total non-protein glutamic acid of hydrolyzed extracts. It represents both free and peptide glutamine and peptide glutamic acid. Glutathione is contained in the growth factor assay medium, and the addition of sterile glutamine to the medium after autoclaving does not change the form of the growth factor curve. Thus, if the similarity of these curves has any special significance, only glutamic acid (or glutamine) peptides other than glutathione could lie at the basis of it.

Investigations bearing on the isolation and identification of these growth factors have shown that they belong to a group of strepogenin-active substances (1). Strepogenin is believed to be a peptide containing glutamic acid (7). The involvement of glutamic acid is also indicated, although indirectly, by these findings. The similarity of the growth factor curve to the free and peptide amino acid curves is not simply an indication that all dialyzable metabolites undergo changes which conform to the same pattern. Studies of vitamins and related growth factors which we are carrying out (1) using the same extracts show that changes of quite a different

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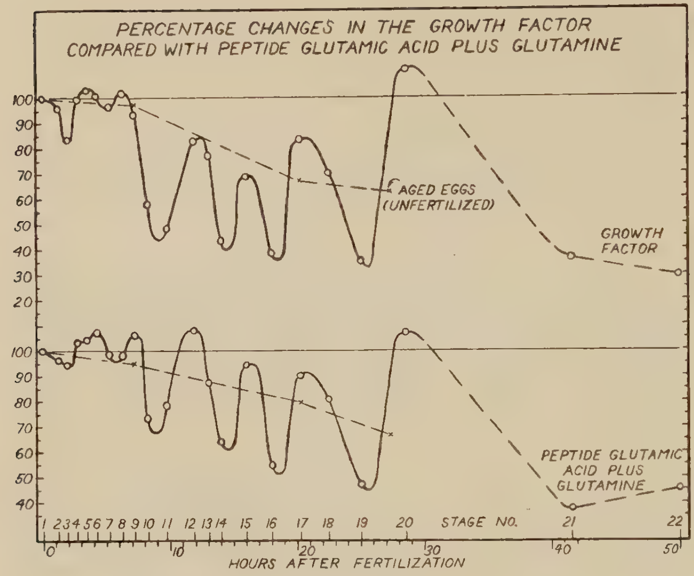

Fig. 1. Percentage changes in growth factor activity compared with the sum of peptide glutamic acid and free glutamine.

nature also occur, although these changes usually come at times which are also critical for the metabolism of amino acids.

It has been found (6) that the changes in the non-protein amino acids are complementary to those of the protein-contained amino acids and appear to be caused by the interplay of yolk breakdown and the synthesis of new embryonic proteins. Since the growth factors change in the same way and appear to be peptides, they may be key yolk components which are incorporated intact into new proteins. When unfertilized eggs are allowed to age, protein synthesis takes place and the non-protein amino acids are depleted (6). Thus, the decrease in growth factors which occurs during the same period probably represents incorporation into the yolk and other proteins.

The growth factor changes found by us are consistent with the relative concentrations at different stages found in aqueous extracts by Gustafson *et al.* (3) insofar as these data can be compared directly. Their curve itself is based upon too few stages to show cyclic changes. The metabolic changes in the sea urchin are now known (6) to be so abrupt and of such short duration that many more stages than had previously been collected are needed and a single highly uniformly developing batch of embryos should be used, if these changes are to be detected.

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MEASUREMENTS OF FEULGEN STAIN DURING THE CELL-CYCLE WITH A NEW PHOTO-ELECTRIC SCANNING DEVICE

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Several methods have previously been described for measuring the amount of stain in cells (9, 11, 6, 13). These methods suffer from the disadvantage that they are sometimes inaccurate and often time-consuming. An instrument is needed which (a) enables the total amount of stain to be measured rapidly so that large quantities of biological material can be surveyed in a short time and (b) increases the accuracy of these measurements so that real variations in stain-content can be more reliably distinguished from variations due to error of measurement (see, for example, (8)).

One of us (EMD) has developed an instrument which fulfils the first requirement and also reduces the errors due to an inhomogeneous distribution of the stain within the measured region (see, for example (2)). In the new instrument the image produced by a microscope is scanned once by a mechanically driven aperture so that only light from successive small elements of the image reach the photomultiplier. This technique has been used in preference to the "flying spot" method, where the light source is a moving spot on one cathode ray tube and an image of the specimen is produced on a second cathode ray tube screen (10); in the present method such an image is not required and monochromatic light of sufficient intensity, which is essential, can be more conveniently obtained from a conventional light source. Light of a wavelength close to that of the maximum absorption of the stain is isolated by filters from a stabilized mercury arc lamp. The signal produced by the photomultiplier circuit, which is designed to give an output proportional to the logarithm of the light intensity, is electrically integrated so as to produce a meter reading having a linear relation to the total amount of absorbing substances. Two measurements are required, one with and one without the specimen in the scanned field, the difference being proportional to the total amount of stain in the specimen. Each measurement of specimen or field is completed in about five seconds.

This instrument is being fully described elsewhere (4) together with appropriate tests of its accuracy and reliability. Fig. 1, however, gives as an example the spread of results obtained when a flat object of low density is measured. Fig. 1a shows the values obtained when one Feulgen-stained sperm head was re-measured 24 times, refocusing and varying the position of the specimen within the field after each measurement. Fig. 1b on the other hand shows the values obtained from 45 different sperm heads in the same preparation. There appears to be no significant difference ($P > 0.5$) between the two distributions, and therefore there can be little real variation in the content of Feulgen stain and hence probably desoxyribose nucleic acid in the measured cells.

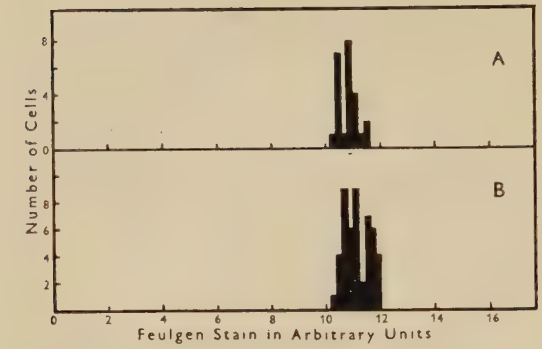
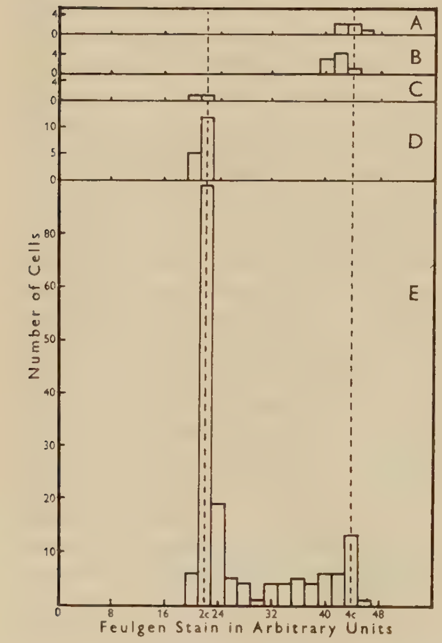


Fig. 1. Measurement of the total Feulgen stain in sperm from the ram.

A. The same sperm head re-measured.
Number of measurements = 24
Mean = 108
Standard deviation = 3.0

B. Different sperm heads from the same field.
Number of measurements = 45
Mean = 111
Standard deviation = 4.3



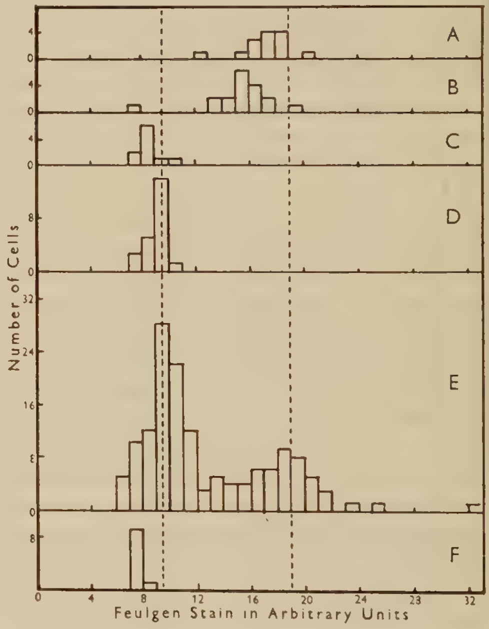


Fig. 2 (left). Measurements of total Feulgen stain in nuclei and mitotic figures from tissue cultures of mouse heart. A = prophase; B = metaphase; C = anaphase; D = telophase; E = interphase.

Fig. 3 (right). Measurements of total Feulgen stain in nuclei and mitotic figures from tissue cultures of chick heart, together with erythrocytes fixed and stained on the same slide. A = prophase; B = metaphase; C = anaphase; D = telophase; E = interphase; F = erythrocytes.

Earlier work in this laboratory has shown that, in contrast to sperm heads and nucleated erythrocytes, interphase nuclei from actively growing tissue cultures show a wide range of values for both the total nuclear absorption at 265 mμ and the total Feulgen stain. This range, which was sometimes bimodal, was shown to be associated with a doubling of the measured material during the latter part of interphase (13). This result supported the conclusions of Swift (11) and of Howard and Pelc (5), that DNA synthesis occurred during interphase, but disagreed with the results from the Brussels school (6) which indicated a synthesis during telophase. These latter results were based primarily on measurements of Feulgen stain during mitotic stages, when errors might be expected to occur in their methods due to inhomogeneity and to the presence of out-of-focus portions of the chromosomes.

Measurements were therefore attempted with the new scanning instrument on both the flattened, lightly absorbing interphase nuclei ($d \sim 0.1$) and the somewhat denser ($d \sim 0.7$) irregular mitotic figures in tissue cultures of mouse and chick embryonic heart. These results are illustrated in Figs. 2 and 3 (each figure being from one slide only). The measurements on interphase nuclei (Figs. 2e and 3e) show the type of distribution that is associated with interphase synthesis. Those from the mouse are particularly interesting (Fig. 2) in that very few lie below the diploid value (Fig. 2C) or above twice that figure (Fig. 4C). This would be expected if there were a constant initial amount of DNA per nucleus which exactly doubles during interphase (12). The wider range in the chick cells may indicate a small real variation in the DNA contents of different nuclei. Turning now to the mitotic stages (Figs. 2 and 3, A–D), it will be seen that while prophase and telophase measurements lie at, or just below, the 4C and 2C means respectively, the values from the metaphase cells are about 20 per cent less than the 4C. A similar departure from the 2c value was found when densely stained avian erythrocytes were measured which had been fixed and stained on the same slide as the chick culture.

It seemed likely that these discrepancies arose from photometric errors due to the high density and three dimensional form of the metaphase chromosomes and the high density and small projected area of the erythrocytes. This was confirmed when, in parallel experiments on the same material, the cells were first "crushed" (3) before measurement in the scanning microscope. After this treatment, which reduces the optical density and brings all parts of the specimen within the depth of focus of the microscope objective, 4c and 2c values for the metaphase cells and erythrocytes respectively were recorded.

These results are interesting for two reasons. First they confirm the conclusions of Alfert and Swift (1) and of Patau and Swift (7), who employed a different technique and different materials, that the synthesis of DNA does not occur during any mitotic stage in the material investigated. They also suggest an explanation of the measurements supporting DNA synthesis during telophase. If a method of measurement is adopted where the photometric errors are somewhat greater than in the scanning instrument used alone without "crushing", it is conceivable that the metaphase measurements could be in fortuitous agreement with the mean of the interphase values, which in many materials may be only a little above the 2C value. Then measurements made on telophase nuclei, in which photometric errors are progressively decreasing as the cells approach interphase, would give a range of readings between half the apparent metaphase and the 2C values.

Second, the results demonstrate that measurements can be speedily and accurately made on relatively large cell populations with the new instrument. It will therefore be possible, by using this instrument and the cell crushing technique, to explore a wider range of tissues than heretofore and to examine how far cells in the body behave in a fashion similar to those grown in tissue culture.

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